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A MESSAGE TO PRACTICING PHARMACISTS

The JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION is published in two editions, the *Practical Pharmacy Edition* and the *Scientific Edition*. All active members of the ASSOCIATION receive both Editions of the JOURNAL each month.

In the *Practical Pharmacy Edition* our readers are kept informed about latest developments in medicine, new prescription products, new techniques and formulations, and important developments along professional and fundamental economic lines.

The *Practical Pharmacy Edition* of THIS JOURNAL is not a trade paper intended to develop merchandising programs or ideas. There are many other publications which fill this important requirement.

Our JOURNAL emphasizes the professional duties and work of the pharmacist and endeavors to give him information and assistance in building his prescription and general pharmaceutical service.

Papers contributed to the Practical Pharmacy Section, the Section on Education and Legislation, the Section on Pharmaceutical Economics, and the Historical Section, are printed in the JOURNAL from time to time when they represent new information or reviews for general practitioners and other important data.

The *Practical Pharmacy Edition* also carries news items of our Local and Student Branches, and about our members who function in various phases of the drug industry. This edition also carries editorial comment on contemporary pharmacy and information on the policy of the ASSOCIATION with respect to current professional and related problems.

Practicing pharmacists have found THIS JOURNAL indispensable in keeping up-to-date and in stimulating their own thinking on problems of the profession and industry.

The *Scientific Edition* of THIS JOURNAL is devoted largely to the publication of papers presented at the annual meetings of the Scientific Section of the ASSOCIATION, and such other articles as are pertinent to scientific progress in pharmacy.

Pharmacists who explore these articles beyond the title often find in the summary, which appears with each paper, something of interest to them and to the members of the medical profession whom they contact in their daily work.

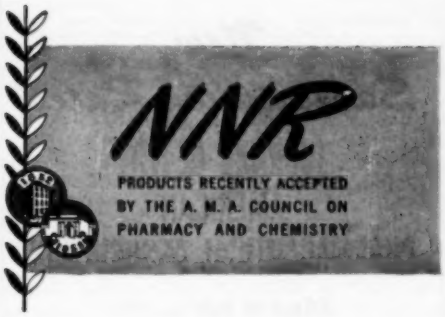
For some months we have been presenting on a single page in the *Scientific Edition* a summary of the advertising which appears in the *Practical Pharmacy Edition* (see page VIII), so that pharmacists who receive the *Scientific Edition* early in the month may be aware of what products or services are being advertised in the *Practical Pharmacy Edition*, which appears later in the month.

The practitioner can, of course, refer to the pages in the *Practical Pharmacy Edition* on which the details of the advertisers' announcements appear.

For some time we have been publishing the text of monographs of "New and Non-Official Remedies" as prepared by the American Medical Association for its annual publication bearing this name, and commonly referred to as N.N.R. These articles are made available in the *Journal of the A.M.A.* from week to week.

By an arrangement with the American Medical Association, this material is furnished to the JOURNAL OF THE A.PH.A. for reprinting in full or in part, and henceforth, this material will appear in the *Scientific Edition* of THIS JOURNAL each month. It will not be in the body of the pages which are usually bound for permanent reference, so that these pages may be cut if the practitioner desires to file them. Furthermore, the N.N.R. material will be printed on one side of the page and will not be backed up by other similar material, so that cutting and filing will be facilitated.

This is an extra service provided by the JOURNAL to practicing pharmacists, and this alone should make the *Scientific Edition* of greater value to the practitioner.



ABSORBABLE GELATIN FILM.—Gelfilm (Upjohn).—A sterile, nonantigenic, absorbable, water-insoluble, gelatin film.—Absorbable gelatin film is obtained by drying on plates at constant temperature and humidity a specially prepared gelatin-formaldehyde solution. It is subsequently sterilized by dry heat at 146–149° for 12 hours.

Actions and Uses.—Absorbable gelatin film is used as an aid in the surgical closure and repair of defects in such membranes as the dura mater and the pleura. It is nonporous and has no hemostatic action. In the dry state absorbable gelatin film has the appearance and stiffness of cellophane of equivalent thickness, but when moistened, assumes a rubbery consistency and can be fitted to rounded, irregular surfaces. Absorbable gelatin film is nonantigenic and does not produce undue inflammatory reaction or other undesirable sequelae.

Dosage.—Absorbable gelatin film, which is approximately 0.075 mm. thick, is applied in the form of sheets. Prior to use the film is soaked in isotonic sodium chloride solution and then cut to the desired shape.

Absorbable gelatin film may be stored indefinitely. To avoid contamination, sterile packages should not be opened until the contents are ready to be applied. When necessary, the film can be resterilized at 140° C. for four hours.

Tests and Standards—

Physical Properties: Absorbable gelatin film is a light yellow, transparent, brittle sheet 0.076–0.228 mm. thick, with a very slight, bouillon-like odor and taste. It is practically insoluble in acetic acid and water. It assumes a rubbery consistency after being in water for a few minutes.

[For more information regarding this monograph see *J. Am. Med. Assoc.*, **153**, 1174 (Nov. 28, 1953).]

The Upjohn Company, Kalamazoo, Mich.

Gelfilm: Box of six absorbable gelatin films in individual sterile envelopes; single films are approximately 100 x 125 x 0.16 mm. U. S. trademark 561,532.

QUININE CARBACRYLIC RESIN.—Diagnex (Squibb).—The quinine salt of a polyacrylic carboxylic acid resin containing about 1.85% of quininium ion.

Actions and Uses.—Quinine carbacrylic resin, a complex of quininium ion and carbacrylic resin, is employed as an indicator for the detection of gastric anacidity (achlorhydria) without intubation. After oral administration of the drug, the quinine in the resin is displaced by the hydrogen ions of free hydrochloric acid that may be present in the stomach. Approximately 1 per cent of the displaced quinine is excreted in the urine within two hours following administration of the resin. A stimulant to gastric secretion is given one hour before administration of the resin. Urine voided during that hour serves as a control sample. Assay of the quinine content of urine specimens, collected at the end of the one-hour control period and two hours after administration of the resin, is performed as an indication of the presence or absence of free hydrochloric acid in the stomach.

The interpretation of the absence or presence of free gastric hydrochloric acid is as follows: Free gastric hydrochloric acid is absent if 15 mcg. of quinine or less is excreted in the two-hour urine specimen. Free gastric hydrochloric acid is present if more than 15 mcg. of quinine is excreted in the two-hour urine specimen. A range of quinine between 15 and 30 mcg. signifies a low degree of gastric acidity.

The quinine carbacrylic resin test does not furnish exact quantitative results; however, it is convenient for screening patients with minor gastric symptoms that are not considered sufficiently significant to warrant the discomfort of intubation gastric analysis or other more expensive diagnostic procedures. The resin test method for achlorhydria is considered useful for the diagnosis of suspected cancer of the stomach, pernicious anemia, and gastric polyps.

Quinine carbacrylic resin is of low toxicity, but it should not be given to patients with an idiosyncrasy to quinine or related drugs.

Dosage.—Quinine carbacrylic resin is administered orally as a single test dose of 2 Gm. in the form of granules. Patients who are given the test should be instructed not to eat after midnight preceding the day of the test. On the morning of the test the patient should empty the bladder and discard the urine. Breakfast is withheld until the test is completed. The contents of a 0.25-Gm. capsule of caffeine and sodium benzoate is stirred and taken in one-half glass of water followed by another one-half glass of water or cup of coffee or tea, without cream, milk, or sugar. One hour later or as soon thereafter as is possible, the patient voids and saves this specimen in a bottle marked "urine control." Then the 2-Gm. dose of the resin is stirred well and taken in one-fourth glass of water (without chewing the granules), followed by another one-fourth glass of water. Exactly two hours after taking the resin, the urine should be voided and the entire amount saved in a bottle marked "urine sample." The bladder should be completely emptied each time; if urination ahead of the scheduled time is necessary, it should be added to that passed at the end of the designated period. After the last specimen is completed, the patient may eat breakfast. The two specimens should be delivered to a clinical laboratory as soon as it is convenient.

If desired, an injection of histamine phosphate may be used in place of oral caffeine as a stimulant to gastric secretion and the control specimen collected after a 45-minute period.

Tests and Standards—

Physical Properties: Quinine carbacrylic resin is a buff, odorless, tasteless, free-flowing, amorphous, granular solid.

(Continued on Page V)

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DILUTION either solution may be diluted with cold (0-5° C.) water or cold buffer solutions of 5.5 to 7.0 pH. Dilute solutions tend to lose activity and should be used same day. Protein materials (i.e. gelatin) tend to impede this inactivation.

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It is practically insoluble in dilute acids and alkalis, alcohol, ether, and water.

[For more information regarding this monograph see *J. Am. Med. Assoc.*, **153**, 1362(Dec. 12, 1953).]

E. R. Squibb & Sons, Division of Mathieson Chemical Corporation, New York.

Granules Diagnex: A quininium indicator resin, each test containing a 2-Gm. packet of quinine carbacrylic resin and a 0.25-Gm. capsule of caffeine and sodium benzoate. U. S. trademark 568,004.

DEXTRAN.—Expandex (Commercial Solvents).—Gentran (Baxter).—Plavolex (Wyeth).—Dextran is a water-soluble, high molecular weight glucose polymer produced by the action of *Leuconostoc mesenteroides* on sucrose. The marketed product has an average molecular weight of about 75,000.

Actions and Uses.—Dextran, when partially hydrolyzed to suitable viscosity and fractionated to provide an average molecular size of 75,000, is useful for intravenous administration in a 6 per cent solution of isotonic sodium chloride to expand plasma volume and maintain blood pressure in emergency treatment of hemorrhagic and traumatic shock. It should neither be regarded as a "substitute" for whole blood or its derivatives essential in restoring blood proteins, nor for combating anemia secondary to hemorrhage or severe traumatic injury such as extensive burns and fractures. Although approximately osmotically equivalent to serum albumin, 6 per cent dextran injection in isotonic sodium chloride solution is not suitable for use in the treatment of edema secondary to hypoproteinemia. Decrease in total serum proteins and hematocrit values, persisting for 6 to 24 hours, is a characteristic response to dextran injection. This is attributed partly to hemodilution, although hematocrit values occasionally return to normal before the total serum proteins. The effect on blood volume of a single injection of 500 to 1,000 cc. of a 6 per cent solution usually persists for 24 hours.

Virtually no adverse reactions have been observed after repeated injections of dextran; however, this polysaccharide has the apparently inherent tendency to produce reactions of an antigen-antibody type in certain human subjects. Such reactions are of low incidence and mild character in adequately hydrolyzed and refined preparations, which provide an average molecular size approximating that of serum albumin. As solutions of dextran do not require refrigeration, they are easily stored and ready for immediate use in emergencies.

Dosage.—Dextran is administered intravenously as a 6 per cent solution in isotonic sodium chloride. The usual dose is 500 cc. infused at the rate of 20 to 40 cc. per minute so that the total amount is administered over a period of about 15 to 30 minutes. Repeated injections may be given when necessary if blood or its derivatives are not available or subsequently indicated. For hemorrhage, the dosage should be limited to an amount sufficient to elevate the systolic blood pressure to not more than 80 to 85 mm. Hg to avoid the production of further bleeding and dangerous dilution of the circulating blood. For shock associated with hemorrhage, severe burns, and traumatic injuries, blood has preference over plasma or artificial colloids.

Abbott Laboratories, North Chicago, Ill.

Solution Dextran 6%. 250- and 500-cc. bottles. The 500-cc. containers are available with or without Venoset (disposable venoclysis unit). An isotonic solution containing 60 mg. of dextran in each cc.

Baxter Laboratories, Inc., Morton Grove, Ill.

Solution Gentran 6%. 250- and 500-cc. bottles. The 500-cc. containers are available with or without disposable syringe. An isotonic solution containing 60 mg. of dextran in each cc.

Commercial Solvents Corporation, New York.

Solution Expandex 6%. 250- and 500-cc. bottles. The 500-cc. containers are available with or without disposable syringe. An isotonic solution containing 60 mg. of dextran in each cc.

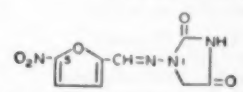
Hyland Laboratories, Los Angeles.

Solution Dextran 6%. 250- and 500-cc. bottles. The 500-cc. containers are available with or without disposable syringe. An isotonic solution containing 60 mg. of dextran in each cc.

Wyeth Laboratories, Inc., Philadelphia.

Solution Plavolex 6%. 500-cc. bottles. An isotonic solution containing 60 mg. of dextran in each cc.

NITROFURANTOIN.—Furadantin (Eaton).—$C_8H_6N_4O_5$.—M.W. 238.16.—N-(5-Nitro-2-furfurylidene)-1-aminohydantoin.—The structural formula of nitrofurantoin may be represented as follows:



Actions and Uses.—Nitrofurantoin, a nitrofuran derivative, exhibits a wide spectrum of antibacterial activity against both Gram-positive and Gram-negative microorganisms. It is bacteriostatic and may be bactericidal to the majority of strains of *Escherichia coli*, *Micrococcus* (*Staphylococcus*) *pyogenes*, *albus*, and *aureus*, *Streptococcus pyogenes*, *Aerobacter aerogenes*, and Paracolobactrum species. The drug is less effective against *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Alcaligenes faecalis*, and Corynebacterium species; many strains of these organisms may be resistant to it; however, bacterial resistance to other anti-infective agents is not usually accompanied by increase in resistance of the organisms to nitrofurantoin. The drug does not inhibit fungi or viruses.

Nitrofurantoin is useful by oral administration for the treatment of bacterial infections of the urinary tract, and its use is indicated in pyelonephritis, pyelitis, and cystitis caused by bacteria sensitive to the drug. The low oral dosage necessary to maintain an effective urinary concentration is not associated with detectable blood levels. The high solubility of nitrofurantoin, even in acid urine, and

(*Continued on page VII*)

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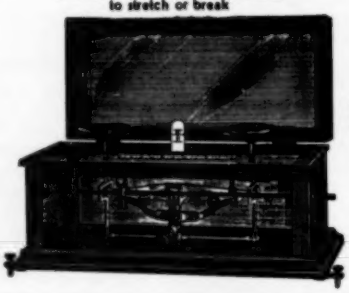

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the low dosage required diminish the likelihood of crystalluria.

Nitrofurantoin has a low toxicity. With oral administration it occasionally produces nausea and emesis; however, these reactions may be obviated by slight reduction in dosage. An occasional case of sensitization has been noted, consisting of a diffuse erythematous maculopapular eruption of the skin. Until more is known concerning its long-term effects, blood cell studies should be made during therapy. Frequent or prolonged treatment is not advised until the drug has received more widespread study. It is otherwise contraindicated in the presence of anuria, oliguria, or severe renal damage.

Dosage.—Nitrofurantoin is administered orally in an average total daily dosage of 5 to 8 mg. per Kg. (2.2 to 3.6 mg. per lb.) of body weight. One-fourth of this amount is administered four times daily—with each meal and with food at bedtime—to prevent or minimize nausea. For refractory infections, such as Proteus and Pseudomonas species, the total daily dosage may be increased to a maximum of 10 mg. per Kg. (4.5 mg. per lb.) of body weight. If nausea is severe, the dosage may be reduced. Medication should be continued for at least three days after sterility of the urine is achieved.

Tests and Standards—

Physical Properties: Nitrofurantoin is a yellow, bitter powder with a slight odor, which decomposes at 258–262°. It is very slightly soluble in alcohol, and practically insoluble in ether and water.

[For more information regarding this monograph see *J. Am. Med. Assoc.*, **154,** 339(Jan. 23, 1954).]

Eaton Laboratories, Inc., Norwich, N. Y.

Tablets Furadantin: 50 and 100 mg. U. S. patent 2,610,181. U. S. trademark 569,968.

NEOMYCIN SULFATE.—Mycifradin Sulfate (Upjohn).—Neomycin is an antibiotic isolated from the elaboration products of *Streptomyces fradiae*, when the microorganism is grown on suitable culture mediums.

Actions and Uses.—Neomycin sulfate is a polybasic compound, thermostable, and soluble in water, but insoluble in organic solvents. It differs from other antibacterial agents in that it is extremely stable and very active in alkaline solution. Neomycin is not inactivated by exudates, enzymes, gastrointestinal secretions, and byproducts of digestion or bacterial growth. The sulfate salt is stable in the dry state for at least two years when stored at room temperature. Prepared solutions retain their potency for at least one year at room temperature, although there may be a progressive deepening of color of solutions stored at room temperature or 37°. Refrigeration of neomycin solutions is, therefore, recommended.

Neomycin sulfate exhibits activity against a variety of Gram-positive and Gram-negative bacteria. In the former group, it appears to be more effective against micrococci (staphylococci) than streptococci. It has a wider antibacterial spectrum than bacitracin, penicillin, or streptomycin, and it is sometimes effective against Pseudomonas and Proteus infections. It is not active against fungi.

Neomycin sulfate is useful for topical applications as a solution or ointment in the local treatment or prevention of susceptible infections of the skin and the eye, including pyogenic or secondarily infected stasis dermatoses, impetigo, wounds, burns, ulcers (varicose or trophic), conjunctivitis, blepharitis, and sty (hordeolum). A solution is considered superior to an ointment in treating trophic ulcers and secondarily infected burns. In severe or extensive infections, local therapy should be supplemented with sulfonamides by mouth or penicillin by injection.

Neomycin sulfate is also useful as an intestinal antiseptic by oral administration for suppression of the usual bacterial inhabitants of the colon in surgery of the large bowel and anus. Because of its poor absorption from the gastrointestinal tract, it rarely produces systemic action or toxic effects when administered orally. Blood levels of 0.2 mg. per cc. may produce serious kidney damage. *Therefore, neomycin should not be injected parenterally, and when given orally, high dosage and prolonged administration should be avoided to prevent possible systemic effects.*

Neomycin sulfate is usually well tolerated and is relatively nonirritating. It is reported to have a low index of sensitization. A mild laxative effect occurs with oral administration. Prolonged oral therapy may result in overgrowth of nonsusceptible organisms—particularly Candida. If new infections caused by bacteria or fungi appear during therapy, it may be advisable to discontinue the drug and/or institute appropriate measures to combat them. Oral use as an intestinal disinfectant is contraindicated in the presence of obstruction.

Dosage.—Neomycin sulfate is applied topically or administered orally for its local action in the large intestine.

For external use, it is applied as a solution containing 5 mg. per cc. or as an ointment containing 5 mg. per Gm. The solution is used for wet dressings, packs, irrigations, or instillations. Topical applications of the solution or ointment are made once or twice daily using an amount sufficient to cover the affected region.

For preoperative disinfection of the colon, the patient is placed on a low residue diet and, immediately after the administration of a cathartic (unless otherwise contraindicated), is given an oral dose of 1 Gm. every hour for four doses followed thereafter by 1 Gm. every four hours for 24 to 72 hours prior to surgery. Administration of the antibiotic should not extend beyond 72 hours. This amount usually produces four to eight bowel movements.

[For more information regarding this monograph see *J. Am. Med. Assoc.*, **154,** 338(Jan. 23, 1954).]

Eli Lilly & Company, Indianapolis.

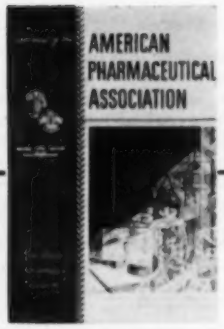
Ointment Neomycin Sulfate. 14.2-, 28.35-, and 113.4-Gm. tubes. An ointment containing 5 mg. of neomycin sulfate in each Gm.

Ophthalmic Ointment Neomycin Sulfate. 3.54-Gm. tubes. An ointment containing 5 mg. of neomycin sulfate in each Gm.

The Upjohn Company, Kalamazoo, Mich.

Powder Mycifradin Sulfate. Vials containing 0.5 Gm. of neomycin sulfate.

Tablets Mycifradin Sulfate. 0.5 Gm.



A Preview of Advertising

In the April 1954 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▸ **Optilets,** multivitamin tablets and their antibiotic **Erythrocin Stearate** are currently featured by **Abbott Laboratories.**

▸ Service, security, and savings are stressed by the **American Druggists' Insurance Company** in its insertion.

▸ **Clinitest,** sealed-in-foil reagent tablets are offered by the **Ames Company** as a "practical, profitable suggestion."

▸ The **Chesebrough Mfg. Co.** emphasizes the dependability of its **Vaseline** sterile petrolatum gauze dressings.

▸ Product information is made easy in **Facts and Comparisons** available through **Drug References.**

▸ **Lactinex** tablets . . . . for reestablishing the normal physiology of the intestinal tract in gastrointestinal disturbances are publicized by **Hynson, Westcott, and Dunning.**

▸ In its insertion, **Eli Lilly and Company** points out the high profit margin **'Homicebrin',** multiple vitamins enjoy.

▸ The **S. E. Massengill Company** lists the following prescription specialties in its announcement: **Adrenosem, Aminodrox, Broniacin, Livitamin, Massengill Powder, Obedrin, Salcedrox,** and **Semhyten.**

▸ Compounding builds prestige, says **Merck & Company,** and observes that its prescription chemicals have helped pharmacists to compound accurately and swiftly for almost a century and a half.

▸ Prescribed to brighten the patient's outlook is **Parke, Davis and Company's** new antidepressant and nutritional adjunct, **Amphedase.**

▸ In two insertions, **Pfizer Laboratories** reiterate the value of **Cortril,** its adrenocortical hormone, and its three Terramycin leaders—**Terramycin Ointment, Terramycin Vaginal Tablets,** and **Terramycin Intramuscular.**

▸ The **Philadelphia College of Pharmacy and Science** offers wide and varied curricula for young people interested in retail, hospital, and manufacturing pharmacy.

▸ **A. H. Robins Company** reports that **Pabalate** and **Pabalate-Sodium Free** tablets provide antirheumatic action closely interrelated with that of the cortisone-like steroids, yet without danger of adverse side effects.

▸ **Tetracyn oral suspension**—"the newest dosage form of the newest broad-spectrum antibiotic"—states **Roerig.**

▸ **Schering** announces its new topical anticholinergic for skin disorders, **Prantal Cream 2%.**

▸ **Sharp and Dohme** suggests **Penalev** to make oral penicillin therapy acceptable to children as well as adults.

▸ **Smith, Kline and French Laboratories** emphasize the ready salability of **Benzedrex Inhalers.**

▸ **Smith-Dorsey** reports that **Rautensin,** for use in the treatment of hypertension, is being intensively promoted to the medical profession.

▸ At the **St. Louis College of Pharmacy and Sciences,** courses in all phases of pharmaceutical work are open to qualified men and women students.

▸ **Panthoderm Cream** is the **U. S. Vitamin Corporation's** contribution for the treatment of dermatoses, eczema, wounds, and burns.

▸ **Warner-Chilcott** offers **Peritrate** to prevent angina pectoris attacks.

▸ Its antiepileptics, **Mebaroin** and **Mebaral,** are publicized in the **Winthrop-Stearns** announcement.

Look for Details in the Practical Pharmacy Edition of This Journal—Out April 20

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIII | *APRIL, 1954* | NUMBER 4 CONSECUTIVE NO. 8

The Synthesis of Some Methyl-Substituted Acridinecarboxylic Acids*

By G. STAPLETON† and A. I. WHITE

Three new methyl-substituted acridinecarboxylic acids have been synthesized in order to test their activity as possible riboflavin antagonists and also to test their possible suppressive action on the growth of microorganisms. The compounds are 7-methylacridine-4-carboxylic acid, 7-methylacridine-2-carboxylic acid, and 6,7-dimethylacridine-2-carboxylic acid. Physical constants have been determined upon them and upon the following new compounds which were synthesized as intermediates: 4-(*p*-methylphenylamino)-isophthalic acid; 7-methylacridone-2-carboxylic acid; 2-chloroisophthalic acid; 2-(*p*-methylphenylamino)-isophthalic acid; 7-methylacridone-4-carboxylic acid; 2-bromo-4,5-dimethylbenzoic acid; 2-(*p*-carboxyphenylamino)-4,5-dimethylbenzoic acid; and 6,7-dimethylacridone-2-carboxylic acid.

SINCE FILDES (1) made the generalization that chemotherapeutic substances might be designed with structures related to essential metabolites or growth factors, there has been an increasing amount of work done toward formulating and synthesizing antimetabolites. Isosterism, a term previously used to describe the fact that two compounds which are closely related in structure and configuration may have the same or similar effects, has often been used to explain the competitive antagonism of antimetabolites. The antagonist is assumed to have a configuration that will fit the "active" points on the enzyme surface but will not react. It thus prevents the enzymatic reaction while it is in place, but it may subsequently be replaced by the metabolite.

Compounds with structures related to the vitamins have proven to be a prolific source of material demonstrating the effect of structural analogs as antimetabolic agents. Various structural modifications of metabolites in this class of compounds have been shown to produce antagonism. A method that often yields excellent results is that in which one ring system is replaced with another. An early example was the substitution of a pyridine ring for the thiazole ring in thiamine, giving pyrithiamine which Robbins (2) reported to inhibit the growth of certain fungi. Later, Wooley and White (3) reported that pyrithiamine produced a characteristic deficiency in mice which could be either prevented or cured by feeding sufficient amounts of thiamine. Recently, pyrithiamine has been shown to be a mixture of the compound it was supposed to represent and of other constituents. The pure drug, which is now called neopyrithiamine, has a much greater inhibitory activity on thiamine-promoted growth than the older mixture (4).

A number of compounds in which the nuclei consist of linear three-ring systems have been shown to inhibit the growth of various microorganisms. It has been suggested that the struc-

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Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.
Portion of a thesis submitted to the Faculty of the Graduate School of the State College of Washington by Gail Stapleton in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmacy.
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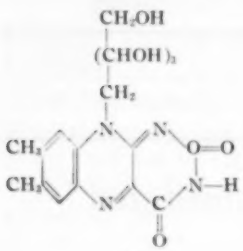
tural relationships of many of these compounds with riboflavin (I) may be responsible for their biological activity.

Birkofer and Birkofer (5) isolated phenazine-1-carboxamide (II) from a culture of *Bacillus pyocyaneus*. The amide was bacteriostatic to *Bacillus anthracis* in 1:200,000 dilution. The phenazine-1-carboxylic acid (III) was one-twentieth as active. Phenazine-1-carboxamide did not inhibit



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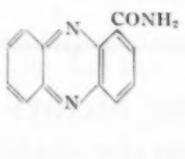

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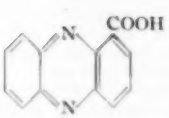

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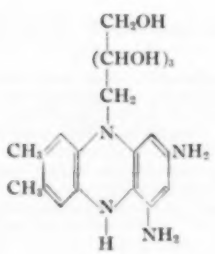

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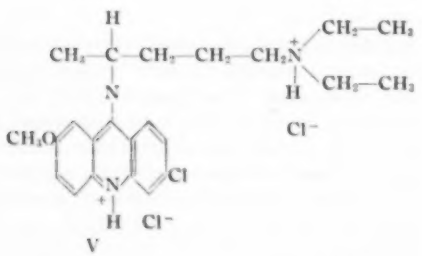

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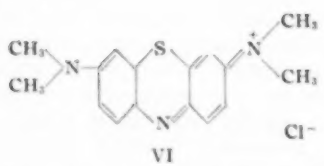


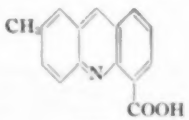
VI

Mycobacterium tuberculosis at 1:10,000 dilution, while phenazine-1-carboxylic acid allowed only a thin growth at this dilution (6). Wooley (7) showed that 2,4-diamino-7,8-dimethyl-9-(*d*-1′-ribityl)-5,10-dihydrophenazine (IV) caused inhibition of growth of certain bacteria, and the inhibition was reversed by riboflavin. The corresponding dinitrophenazine was not inhibitory to the growth of bacteria, but produced a mild riboflavin deficiency when fed to mice.

Madinaveitia (8), using *Lactobacillus casei* as the test organism, reported that, among other drugs, quinacrine hydrochloride [3-chloro-7-methoxy - 9 - (1 - methyl - 4 - diethylaminobutylamino)-acridine dihydrochloride] (V) and methylene blue (tetramethyl-thionine chloride) (VI) inhibited the activity of riboflavin. Hellerman, Lindsay, and Bovarnick (9) found that quinacrine was one of a number of drugs that inhibit *d*-amino acid oxidase, a flavin enzyme. They suggested, however, that the inhibition did not appear to be truly competitive.

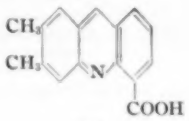
After a study of the three-ring compounds previously mentioned, it was decided that it would be of interest to synthesize some substituted acridines which would resemble the riboflavin ring nucleus in general structure and in the position of some of the substituents. To accomplish

VII



VIII

IX

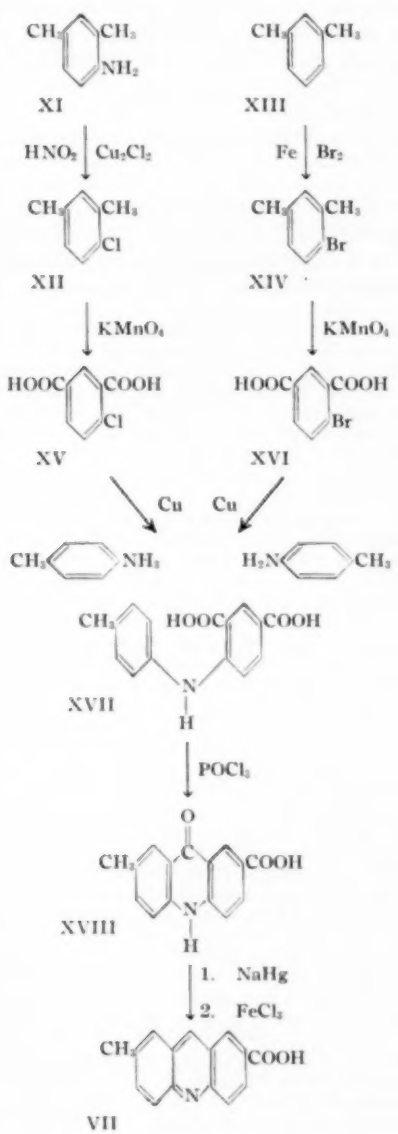


X

this, it was decided to prepare some methyl-substituted acridinecarboxylic acids. The carboxyl group was to be in the 2- or 4-position, corresponding to one of the keto groups in riboflavin. In the monomethyl compounds the methyl group was to be on the 7-carbon. The 7- and 8-methyl-9-(*d*-1′ribityl)-isoalloxazines have approximately one-half the vitamin activity of riboflavin (10). The dimethyl acridinecarboxylic acid was to have the methyl groups in the 6- and 7-positions and the carboxyl group in the 2-position. The compounds that have been synthesized are 7-methylacridine-2-carboxylic acid (VII), 7-methylacridine-4-carboxylic acid (VIII) and 6,7 - dimethylacridine - 2 - carboxylic acid (IX). The synthesis of 6,7-dimethylacridine-4-carboxylic acid (X) is planned for a later date.

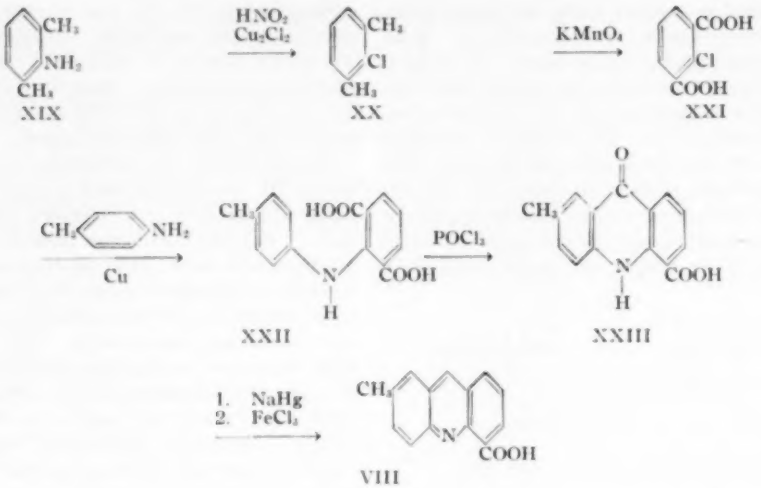
The synthesis of 7-methylacridine-2-carboxylic acid (VII) was accomplished in six steps, either starting with 4-amino-1,3-dimethylbenzene (XI) and converting it to 4-chloro-1,3-dimethylbenzene (XII) by the Sandmeyer reaction, or by starting with *m*-xylene (XIII) and brominating (11) to 4-bromo-1,3-dimethylbenzene (XIV). The sec-

ond method gave better yields and larger quantities could be handled more conveniently. Both of the 4-halo-1,3-dimethylbenzenes were oxidized with potassium permanganate to the corresponding 4-chloroisophthalic acid (XV) or 4-bromoisophthalic acid (XVI). The oxidations proceeded slowly, but the yields were satisfactory. The haloisophthalic acids were condensed with *p*-toluidine using the Jourdan-Ullmann reaction to form 4-(*p*-methylphenylamino)-isophthalic acid (XVII). Ring closure to 7-methylacridone-2-carboxylic acid (XVIII) was effected easily by using phosphorus oxychloride. Sodium amalgam was used to reduce the acridone to the acridan, which was not isolated. Oxidation to 7-methylacridine-2-carboxylic acid (VII) was accomplished in moderate yields with ferric chloride.



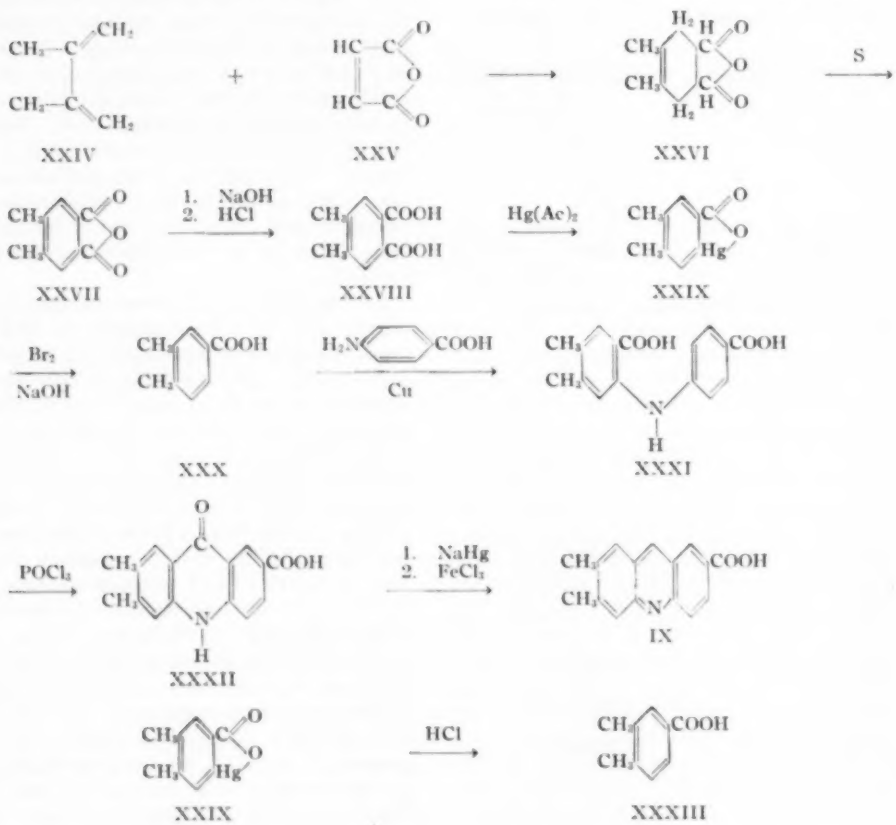
The synthesis of 7-methylacridine-4-carboxylic acid (VIII) was accomplished in much the same manner as that for 7-methylacridine-2-carboxylic acid. Using the Sandmeyer method, 2-amino-1,3-dimethylbenzene (XIX) was transformed into 2-chloro-1,3-dimethylbenzene (XX) which was then oxidized to 2-chloroisophthalic acid (XXI) with potassium permanganate. The oxidation took place more readily than did the oxidation of 4-chloro-1,3-dimethylbenzene, although the yields were nearly the same. No description of 2-chloroisophthalic acid could be found in the literature, although 2-bromoisophthalic acid (12) and 2-iodoisophthalic acid (13) have been reported. The condensation with *p*-toluidine to form 2-(*p*-methylphenylamino)-isophthalic acid (XXII) required less rigorous conditions than the condensation involving 4-chloroisophthalic acid. The ease of reaction more nearly paralleled that of 4-bromoisophthalic acid with *p*-toluidine. Ring closure to 7-methylacridone-4-carboxylic acid (XXIII) was accomplished readily and in similar yields to the closure involving 4-(*p*-methyl-phenylamino)-isophthalic acid. Reduction of 7-methylacridone-4-carboxylic acid to the acridan, which again was not isolated, and oxidation of the acridan to the 7-methylacridine-4-carboxylic acid (VIII) were carried out in the same manner as for 7-methylacridone-2-carboxylic acid.

The synthesis of 6,7-dimethylacridine-2-carboxylic acid (IX) was accomplished from 2-bromo-4,5-dimethylbenzoic acid (XXX) and *p*-amino-benzoic acid by following the same general procedures as for the two monomethyl acridine-carboxylic acids. For the preparation of 2-bromo-4,5-dimethylbenzoic acid, 2,3-dimethylbutadiene-1,3 (XXIV) was condensed with maleic anhydride (XXV) in a Diels-Alder reaction, yielding 4,5-dimethyl-1,2,3,6-tetrahydrophthalic anhydride (XXVI) (14) in excellent yields. The 4,5-dimethyl-1,2,3,6-tetrahydrophthalic anhydride was dehydrogenated to 4,5-dimethylphthalic anhydride (XXVII) using sulfur, in a modification of the method used by Sugasawa and Sugimoto (15). Attempts to obtain a crystalline product, using the temperature of 240° indicated by them failed. However, by lowering the temperature to 205–215°, 4,5-dimethylphthalic anhydride was obtained in yields of 50–55 per cent. The crystals were contaminated with sulfur to a



small extent but were sufficiently pure for the subsequent reactions. The anhydride was hydrolyzed to 4,5-dimethylphthalic acid (XXVIII) by refluxing in dilute sodium hydroxide solution.

Anhydro - 2 - hydroxymercuri - 4,5 - dimethylbenzoic acid (XXIX) was prepared by mercuration of 4,5-dimethylphthalic acid with mercuric acetate (16). The mercury was replaced with



bromine (17) to yield 2-bromo-4,5-dimethylbenzoic acid (XXX). Condensation of the 2-bromo-4,5-dimethylbenzoic acid with *p*-aminobenzoic acid yielded 2-(*p*-carboxyphenylamino)-4,5-dimethylbenzoic acid (XXXI) which was cyclized with phosphorus oxychloride to 6,7-dimethylacridone-2-carboxylic acid (XXXII). Reduction of the acridone with sodium amalgam and oxidation with ferric chloride gave 6,7-dimethylacridine-2-carboxylic acid (IX).

The position of the mercury atom of the anhydro - 2 - hydroxymercuri - 4,5 - dimethylbenzoic acid was verified by heating the compound with 36 per cent hydrochloric acid and obtaining 3,4-dimethylbenzoic acid (XXXIII).

EXPERIMENTAL

4 - Chloro - 1,3 - dimethylbenzene.—Seventy-two grams (0.6 mole) of 4-amino-1,3-dimethylbenzene (Eastman) was added slowly and with stirring to a solution of 128 ml. of 36% hydrochloric acid and 128 ml. of water. The slurry of fine crystals was placed in an ice-salt bath and diazotized at a temperature of 3–8° with an aqueous solution of 44 Gm. of sodium nitrite. During the reaction, cracked ice was added to the reaction mixture. The reaction mixture was slowly poured into a solution of 60 Gm. of cuprous chloride (analytical reagent) in 350 ml. of 36% hydrochloric acid. The cuprous chloride solution had been previously warmed to 40–50°, and after the addition of the diazotized solution heat was applied to raise slowly the temperature to 70°. After cooling to room temperature, the red-brown oil was removed by means of a separatory funnel and washed with cold concentrated sulfuric acid and two portions of water. The oil was distilled at atmospheric pressure (698.5 mm.) and the fraction boiling between 182° and 184° collected. The yield was 36 Gm. (42%). Klages (18) reported the boiling point of 4-chloro-1,3-dimethylbenzene as 187–188° at 755 mm.

4-Chloroisophthalic Acid.—Fourteen grams (0.1 mole) of 4-chloro-1,3-dimethylbenzene in 2 L. of water was heated, with stirring, just below the reflux temperature for a total of thirty-five hours on three successive days. During the heating, 100 Gm. of potassium permanganate was added in 10- to 15-Gm. portions. While the reaction mixture was still warm, 95% ethanol was added until the excess permanganate was destroyed. After cooling to room temperature, the manganese dioxide was removed by filtration with suction. The cake was washed with several portions of water and sucked as dry as possible. The filtrate was acidified with 36% hydrochloric acid, filtered, and the precipitate dried at 100°. The 4-chloroisophthalic acid was crystallized from a large volume of water. The yield of crystalline product was 11.5 Gm. (58.5%) melting at 293–294°. Ullmann and Uzbachian (19) reported the melting point of 4-chloroisophthalic acid as 294.5°.

4-Bromoisophthalic Acid.—Twenty-seven and seven-tenths grams (0.15 mole) of 4-bromo-1,3-dimethylbenzene (11) in 2 L. of water was heated, with stirring, for a total of forty-eight hours over a six-day period. One hundred and fifty grams of potassium permanganate was added in 10- to 15-Gm. portions during the stirring. After destroying the excess permanganate with 95% ethanol, filtering and then precipitating the 4-bromoisophthalic acid with 36% hydrochloric acid, the compound was crystallized from an ethanol-water mixture. The yield was 19 Gm. (52%) melting at 281°. Schöpff (20) reported the melting point of 4-bromoisophthalic acid as 283°.

4-(*p*-methylphenylamino)-isophthalic Acid.—(*A*) A mixture of 8 Gm. (0.04 mole) of 4-chloroisophthalic acid, 11 Gm. of anhydrous potassium carbonate, 6.4 Gm. (0.6 mole) of *p*-toluidine and 0.2 Gm. of activated copper (21) in 40 ml. of 4-methylcyclohexanol was refluxed, with stirring, for eight hours. The 4-methylcyclohexanol was removed by steam distillation and the solution filtered, while hot, from the insoluble residue. The filtrate was then boiled with charcoal, filtered, heated again to boiling, and the 4-(*p*-methylphenylamino)-isophthalic acid precipitated with 36% hydrochloric acid. The precipitate was immediately collected from the hot solution on a Büchner funnel. The cake was dried at 100°. The acid was partially purified by allowing it to precipitate from hot ethanol-water solution. The amorphous material was sufficiently pure for the subsequent step. The yield was 7.2 Gm. (66%). For analysis, the compound was crystallized by dissolving it in ethyl-methyl ketone with the aid of heat and rapidly reducing the volume of the solution by boiling it until cloudiness just appeared. Crystallization occurred on cooling. The melting point of recrystallized 4-(*p*-methylphenylamino)-isophthalic acid was 285.5–286°, corrected, with decomposition.

Anal.[1]—Calcd. for $C_{15}H_{13}NO_4$: C, 66.41; H, 4.83; N, 5.16. Found: C, 66.92; H, 4.93; N, 5.09.

(*B*) A mixture of 9.8 Gm. (0.04 mole) of 4-bromoisophthalic acid, 11 Gm. of anhydrous potassium carbonate, 6.4 Gm. (0.06 mole) of *p*-toluidine and 0.2 Gm. of activated copper (21) in 40 ml. of *n*-butanol was refluxed for six hours. Beginning with the steam distillation of the *n*-butanol, the procedure was the same as that used for the condensation of 4-chloroisophthalic acid with *p*-toluidine. The yield was 8 Gm. (74%) of product melting at 284–285° after recrystallization from ethyl-methyl ketone.

7-Methylacridone-2-carboxylic Acid.—Five and four-tenths grams (0.02 mole) of 4-(*p*-methylphenylamino)-isophthalic acid was covered with 16 ml. of phosphorus oxychloride and heated slowly until the vigorous reaction had subsided and solution had taken place. The solution was refluxed for two hours and excess phosphorus oxychloride removed by vacuum distillation until a thick syrup remained. Forty milliliters of 1 *N* hydrochloric acid was slowly and carefully added to the residue. A vigorous exothermic reaction took place. The mixture was heated for six to eight hours at 60–80°. Once or twice during the heating period the material adhering to the sides of the flask was loosened by scraping it free. The granular solid was removed from the reaction mixture by filtration and dried

[1] Analyses were performed either by Clark Microanalytical Laboratory, Urbana, Illinois, or by Microchemical Specialties Co., Berkeley, California.

at 100°. The 7-methylacridone-2-carboxylic acid was boiled with about 200 ml. of ethanol to remove any unchanged starting material. There was no solvent found in which the acridonecarboxylic acid was sufficiently soluble so that it could be crystallized. The residue after boiling with ethanol, however, was quite pure. In attempting to take a melting point the compound was heated to 500°. There was slow decomposition but no discernible melting point. The yield was 4.3 Gm. (86%).

Anal.—Calcd. for $C_{15}H_{11}NO_3$: C, 71.14; H, 4.38; N, 5.53. Found: C, 70.96; H, 4.32; N, 5.64.

7-Methylacridine-2-carboxylic Acid.—The procedure was adapted from that used by Albert and Goldacre (22) for the preparation of acridine-2-carboxylic acid.

Two and five-tenths grams (0.01 mole) of 7-methylacridone-2-carboxylic acid in 25 ml. of 0.5 *N* sodium hydroxide was heated to 80°, with the use of an efficient semicircular stirrer that just touched the bottom of the flask. During a period of one and one-half to two hours, 46 Gm. of 2.5% sodium amalgam was added in small portions. During the addition a precipitate formed. Heating and stirring were then continued for an additional hour. The reaction mixture was then diluted to about 400 ml. with water, heated to boiling to dissolve the precipitated solid, and filtered. The filtrate was made acid to methyl orange with 36% hydrochloric acid and boiled for two hours with 13 Gm. of ferric chloride. The ferric chloride was added in the form of a concentrated aqueous solution. The reaction mixture was made alkaline with sodium carbonate. Hyflo Super-Cel® (Johns-Manville) filteraid was added and the mixture heated to boiling and then filtered. The cake was washed with several portions of boiling dilute sodium carbonate solution. The filtrate was cooled to room temperature and acidified with 36% hydrochloric acid. The precipitated 7-methylacridine-2-carboxylic acid was removed by filtration, dried at 100°, and crystallized from a methyl cellosolve-water mixture. The yield was 1.2 Gm. (63%) of yellow crystals melting at 304–306° with decomposition.

Anal.—Calcd. for $C_{15}H_{11}NO_4$: C, 75.93; H, 4.67; N, 5.90. Found: C, 76.17; H, 5.23; N, 5.98.

2-Chloro-1,3-dimethylbenzene.—Seventy-two grams (0.6 mole) of 2-amino-1,3-dimethylbenzene (Eastman) was diazotized according to the procedure described for the diazotization of 4-amino-1,3-dimethylbenzene. The reaction mixture was poured slowly, with stirring, into a solution of 60 Gm. of cuprous chloride (analytical reagent) in 350 ml. of 36% hydrochloric acid. The temperature of the cuprous chloride solution was raised to 30° before adding the diazotized 2-amino-1,3-dimethylbenzene. After the addition, the temperature had increased to 50°. The mixture was heated to 80° by the application of external heat in order to complete the reaction. Purification of the 2-chloro-1,3-dimethylbenzene was accomplished in the same manner as that for 4-chloro-1,3-dimethylbenzene. The yield was 38 Gm. (46%) of a liquid boiling between 181° and 183° at 701.9 mm. pressure. Kohlrausch and Pongratz (23) reported the boiling point of 2-chloro-1,3-dimethylbenzene as 183.5–185.5°.

2-Chloroisophthalic Acid.—Fourteen grams (0.1 mole) of 2-chloro-1,3-dimethylbenzene in 2 L. of water was stirred and heated below the reflux temperature for a total period of twenty-one hours on three successive days. During the heating period, 100 Gm. of potassium permanganate was added in 5- to 10-Gm. portions. While the reaction mixture was still warm, sufficient 95% ethanol was added to destroy the excess permanganate. After cooling the mixture to room temperature, the manganese dioxide was filtered off with the aid of suction, the cake washed with several portions of water and then sucked as dry as possible. The filtrate was acidified with 36% hydrochloric acid, the precipitate removed by filtration, and dried at 100°. The yield of 2-chloroisophthalic acid after one crystallization from water was 12 Gm. (60%) melting at 223–223.5°, corrected.

Anal.—Calcd. for $C_8H_5ClO_4$: Cl, 17.68. Found: Cl, 17.61.

2-(*p*-methylphenylamino)-isophthalic Acid.—A mixture of 8 Gm. (0.04 mole) of 2-chloroisophthalic acid, 11 Gm. of anhydrous potassium carbonate, 6.4 Gm. (0.06 mole) of *p*-toluidine and 0.2 Gm. of activated copper (21) in 40 ml. of *n*-butanol was refluxed, with stirring, for six hours. Steam distillation of the solvent and purification was accomplished in the same manner as for 4-(*p*-methylphenylamino)-isophthalic acid. The yield of almost pure 2-(*p*-methylphenylamino)-isophthalic acid was 7.2 Gm. (66%). After recrystallization from ethanol-water, the melting point was 214–215°, corrected, with decomposition.

Anal.—Calcd. for $C_{16}H_{13}NO_4$: C, 66.41; H, 4.83; N, 5.16. Found: C, 66.53; H, 4.93; N, 5.31.

7-Methylacridone-4-carboxylic Acid.—The same procedure used for the cyclization of 4-(*p*-methylphenylamino)-isophthalic acid was used. The yield from 5.4 Gm. (0.02 mole) of 2-(*p*-methylphenylamino)-isophthalic acid, after crystallization from approximately 600 ml. of ethanol, was 4.2 Gm. of yellow crystals melting at 325–327° with decomposition.

Anal.—Calcd. for $C_{15}H_{11}NO_3$: C, 71.14; H, 4.38; N, 5.53. Found: C, 71.45; H, 4.38; N, 5.18.

7-Methylacridine-4-carboxylic Acid.—The procedure was similar to that used for the reduction and oxidation of 7-methylacridone-2-carboxylic acid. Two and five-tenths grams (0.01 mole) of 7-methylacridone-4-carboxylic acid was heated for four hours while 74 Gm. of 2.5% sodium amalgam was added in small portions. After one-third to one-half of the sodium amalgam had been added, a precipitate formed which redissolved toward the end of the addition. The alkaline filtrate from the ferric chloride oxidation procedure was made acid with 36% hydrochloric acid, heated to boiling, and filtered. On cooling, crystals separated from the filtrate. After drying at 100°, the 7-methylacridine-4-carboxylic acid was recrystallized from ethanol-water. The yield was 0.9 Gm. (38%) of bright yellow needles melting at 206–207°, corrected.

Anal.—Calcd. for $C_{15}H_{11}NO_3$: C, 75.93; H, 4.67; N, 5.90. Found: C, 76.04; H, 4.72; N, 5.81.

4,5-Dimethyl-1,2,3,6-tetrahydrophthalic Anhydride.—The Diels-Alder condensation of 2,3-dimethylbutadiene-1,3 was carried out according to the procedure of Farmer and Warren (14). The anhydride was obtained from the reaction mixture by heating it to boiling and adding petroleum ether, with stirring, until crystallization took place. The

hydroquinone antioxidant present in the dimethylbutadiene (obtained from Monomer-Polymer, Inc., of Leominster, Mass.) was not removed before the condensation. It should be noted that the reaction is vigorously exothermic and the reaction mixture should be cooled after the reaction begins and cooling continued until it moderates.

4,5-Dimethylphthalic Anhydride.—The dehydrogenation of 4,5-dimethyl-1,2,3,6-tetrahydrophthalic anhydride, by means of sulfur, to 4,5-dimethylphthalic anhydride was similar to that used by Sugasawa and Sugimoto (15) except for the temperature. Twenty-one and six-tenths grams (0.12 mole) of the tetrahydrophthalic anhydride was heated with 8 Gm. (0.25 Gm. atoms) of sulfur for a period of two to two and one-half hours in an oil bath with the bath temperature maintained at 205–215°. The flask was immersed to the neck in the oil bath. The 4,5-dimethylphthalic anhydride was crystallized by adding benzene, heating to boiling, and filtering the benzene solution through a heated funnel. On cooling the solution to room temperature, crystals of 4,5-dimethylphthalic anhydride separated. The yield was 12 Gm. (57%) of crystals slightly contaminated with sulfur but sufficiently pure for subsequent steps.

2-Bromo-4,5-dimethylbenzoic Acid.—The procedure was adopted from that used for the preparation of anhydro-2-hydroxymercuri-3-nitrobenzoic acid (16) and its subsequent bromination to 2-bromo-3-nitrobenzoic acid (17).

A solution of 4 Gm. (0.1 mole) of sodium hydroxide in 40 ml. of water was prepared in a 100-ml. beaker. To the warm solution was added 9.7 Gm. (0.05 mole) of 4,5-dimethylphthalic acid (obtained by hydrolyzing the anhydride with dilute sodium hydroxide). A solution of 17.5 Gm. (0.055 mole) of mercuric acetate in a mixture of 2.5 ml. of glacial acetic acid and 35 ml. of water was prepared by heating and filtering while hot. The two hot solutions were poured into a 300-ml. flask and mixed by shaking. A heavy white precipitate separated. The flask was clamped in an oil bath with the level of the oil up to the neck of the flask. The flask was fitted with a reflux condenser provided with a tube leading to the desk. The temperature of the oil bath was raised to 170° over a period of about one hour. The heating was continued for eight to ten hours at 165–175°. After a few hours the end of the tube leading from the condenser was introduced below the surface of a few milliliters of heavy mineral oil in a small beaker. When not more than one bubble every four or five minutes escaped, the heating was discontinued. The product was filtered through a suction filter and dried at 100°. No attempt was made to purify the compound.

A solution of 2.5 Gm. (0.62 mole) of sodium hydroxide in 75 ml. of water was prepared in a 250-ml. flask and the solution heated to gentle boiling. The anhydro-2-hydroxymercuri-4,5-dimethylbenzoic acid was added in portions of 2 to 2.5 Gm., with stirring, until the material had gone into solution except for a small residue. There was slowly added, with continuous stirring, 5 Gm. of 36% hydrochloric acid. Heating was discontinued at this point and 1.6 Gm. of glacial acetic acid was added slowly. A curdy precipitate formed. A solution of bromine was prepared by dissolving 5.15 Gm. (0.05 mole) of sodium bromide and 8 Gm. (0.05 mole) of bromine in 7.5 ml. of water. The mixture was stirred and the bromine solution was added as rapidly as possible through an addition funnel. The mixture was heated to boiling for five minutes after the last of the bromine had been added, was made alkaline by the addition of 1 Gm. of sodium hydroxide, and was filtered. The filtrate was made acid to congo red paper by the addition of 36% hydrochloric acid and the precipitate removed by filtration. After drying at 100° and crystallizing from an ethanol-water mixture, the yield was 5.6 Gm. (49%) of white crystals melting at 195–196°, corrected.

Anal.—Calcd. for $C_9H_9BrO_3$: C, 47.18; H, 3.96; Br, 34.89. Found: C, 47.04; H, 4.0; Br, 34.99.

2-(*p*-carboxyphenylamino)-4,5-dimethylbenzoic Acid.—The same procedure as that used for the preparation of 2-(*p*-methylphenylamino)-isophthalic acid and 4-(*p*-methylphenylamino)-isophthalic acid was followed.

Six and nine-tenths grams (0.03 mole) of 2-bromo-4,5-dimethylbenzoic acid, 6.3 Gm. (0.045 mole) of *p*-aminobenzoic acid, 8.3 Gm. of anhydrous potassium carbonate and 0.2 Gm. of activated copper (21) were refluxed in 50 ml. of *n*-butanol for five hours. After the preliminary purification and crystallization from ethanol-water, the yield was 5.9 Gm. (70%) melting at 273.5–274°, corrected, with decomposition.

Anal.—Calcd. for $C_{16}H_{15}NO_4$: C, 67.35; H, 5.30; N, 4.91. Found: C, 67.14; H, 5.48; N, 4.83.

6,7-Dimethylacridone-2-carboxylic Acid.—The same procedure as that used for the preparation of 7-methylacridone-2-carboxylic acid and 7-methylacridone-4-carboxylic acid was followed.

The yield of 6,7-dimethylacridone-2-carboxylic acid from 5.7 Gm. (0.02 mole) of 2-(*p*-carboxyphenylamino)-4,5-dimethylbenzoic acid was 4.7 Gm. (84%). It was partially purified by heating with ethanol. On heating the compound to 500° there was slow decomposition but no discernible melting point.

Anal.—Calcd. for $C_{16}H_{13}NO_3$: C, 71.88; H, 4.90; N, 5.24. Found: C, 71.73; H, 5.04; N, 5.29.

6,7-Dimethylacridine-2-carboxylic Acid.—The reduction and oxidation procedures used for the preparation of 7-methylacridine-2-carboxylic acid and 7-methylacridine-4-carboxylic acid were followed. For the reduction, a total of 40 Gm. of 2.5% sodium amalgam was added to a hot solution of 1.6 Gm. (0.006 mole) of 6,7-dimethylacridone-2-carboxylic acid over a period of two hours. Heating was continued for another hour. Crystallization was quite difficult but could usually be accomplished by dissolving the crude acid in hot ethanol, adding hot water to the point of cloudiness, and allowing it to precipitate in the refrigerator. The amorphous solid could then be crystallized from a methyl cellosolve-water mixture. The yield was 360 mg. (23%) of crystals melting at 312–313° with decomposition.

Anal.—Calcd. for $C_{16}H_{13}NO_2$: C, 76.48; H, 5.22; N, 5.57. Found: C, 76.61; H, 5.38; N, 5.50.

3,4-Dimethylbenzoic Acid.—Ten grams of anhydro-2-hydroxymercuri-4,5-dimethylbenzoic acid was heated with 40 ml. of 36% hydrochloric acid for one hour. The mixture was filtered, the precipitate suspended in 200 ml. of water and made alkaline with solid sodium hydroxide. The mixture was again filtered and the filtrate made acid with

36% hydrochloric acid. The resulting precipitate was removed by filtration and crystallized from ethanol-water. After drying at 100° the crystals melted at 166.5°. Lellmann and Just (24) reported the melting point of 3,4-dimethylbenzoic acid as 166°.

2-Methylacridine.—7-Methylacridine-2-carboxylic acid and 7-methylacridine-4-carboxylic acid were both decarboxylated by the same procedure to yield 2-methylacridine, a known compound.

Two hundred milligrams of the methylacridinecarboxylic acids were heated to 300° in a sulfuric acid bath for five to ten minutes. The residue was extracted with dilute sodium hydroxide solution and crystallized from ethanol-water. The resulting crystals melted at 134–134.5°. Reed (25) reported the melting point of 2-methylacridine to be 134°.

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The Use of Certain Rye Homogenates for Supporting Growth of *Claviceps purpurea* (Fries) Tulasne*

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The growth of a known strain of *Claviceps purpurea* on several homogenates of three different stages of rye inflorescence has been compared qualitatively. The liquid phase of the filtered homogenates supported the best growth, and most satisfactory results were achieved with the use of fruiting inflorescences. Growth was greatly inhibited in all conditions in the absence of chlorophyll.

NUMEROUS ATTEMPTS have been reported (1–7) on the growth of *Claviceps purpurea* and its various strains with the use of artificial surface and submerged cultures. In general, these have been successful to the extent of the production of mycelial tissues and conidiospore growth. Several of these workers (2–7) have obtained growth on semisynthetic media as well. For example, nutrient substrates containing sucrose, maltose, levulose, mannitol, peptone, yeast extract, various inorganic salts, etc., have been employed. Bonns (1) has with some success investigated the use of more natural media such as mashes of rye seed, rye screening, and rye heads after flowering; while Michener (5) has employed homogenates of germinated wheat in his studies of *Claviceps* growth and Tyler (7) and Kreitmair (3) have utilized homogenated young rye tissues apart from the inflorescence stage. The results of growth and alkaloid formation under these conditions have been reported with varying success.

In the course of studies in this laboratory of nutritional factors present in the rye, which under normal conditions support the *Claviceps* life cycle, it appeared of interest to examine more critically several homogenates prepared from different stages of the entire rye inflorescence as substrates for the growth of this fungus in submerged culture and to determine the efficiency of these for this purpose. This report describes the comparative growth response of one active strain of *Claviceps* on cultures prepared with these homogenates.

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EXPERIMENTAL

The experiment was arranged so that several modifications of each of three stages of the rye inflorescence: (*a*) immature or nonflowering, (*b*) flowering or anthesis, and (*c*) mature or fruiting, could be critically studied for *Claviceps* growth. Each of these three stages was treated so that frozen, fresh, dried, chlorophyll, and chlorophyll-less conditions could be examined. Further, both mash and liquid phases of each homogenate were included.

Immature, flowering, and mature rye heads, collected from a cultivated plot of rye *Secale cereale* at Monroe, Washington, in June and July, 1952, and at the University of Washington Drug Plant Garden in August, 1952, and June and July of 1953, were frozen, or processed immediately, or dried for twenty-four hours at about 46°. Recognition of these stages was made by a macroscopic inspection of the inflorescence. The immature stage was identified by the presence of well-formed glumes and awns. Ovarian tissues were formed, but stamens were immature. The flowering stage was recognized by the presence of mature anthers which covered the entire inflorescence. In some cases, the stigma were also exposed. The grain stage was identified by the presence of many caryopses in the spikelets, each enclosed within palets. An authentic strain of *Claviceps purpurea* which had been originally obtained from the laboratory of Dr. E. C. Stakman, Department of Plant Pathology, University of Minnesota, was employed throughout the experiment as the *Claviceps* strain. Stock cultures of this strain were maintained on sterile agar slants prepared with the formula of McCrea (2). Transplants were made from the original agar cultures every four weeks.

Fifty-gram portions of frozen and fresh rye heads were cut into small fragments and blended in a Waring Blendor with 500 ml. of distilled water for five minutes. The green suspension and the rye head mash were separated by passing the homogenate through an ordinary kitchen strainer. The strained rye head mash was either extracted with acetone to remove chlorophyll and used as a culture medium or immediately transferred in 20-Gm. (wet weight) portions to several 500-ml. Erlenmeyer or 60-ml. Kolle flasks (diameter 13.5 cm.). Distilled water was added to make the total volume in each flask 100 cc. and 60 cc., respectively. The green suspension which had been separated from the rye head mash was then divided into aliquots as follows for growth study: One aliquot was transferred directly into culture flasks and employed as the growth medium. A second aliquot was centrifuged at 1,500 r. p. m. in an International centrifuge apparatus for ten minutes. The centrifuged liquid was then filtered through Eaton and Dickson No. 613 paper and 100 ml. of the resultant yellow-green filtrate was then dispensed into Erlenmeyer flasks and 60 ml. placed into Kolle flasks. A third aliquot of the green suspension was extracted with ether until chlorophyll was removed. The extracted liquid which then became brown-amber in color was similarly transferred to Erlenmeyer and Kolle flasks. All growth cultures were plugged with sterile cotton and made ready for sterilization. Two methods were selected for sterilization: a conventional autoclaving process during which steam at 15 lbs. pressure for twenty minutes was employed for one batch and a chemical procedure after the method of Hansen and Synder (8) using propylene oxide (Eastman Kodak Co.) for the second batch. In the latter method of sterilization the culture media were introduced into flasks whereupon these were plugged with cotton and placed into a 10-L. metal container which was surrounded with ice. Two flasks were placed in each container with 10 ml. of propylene oxide. The container was immediately sealed so as to make it airtight. At room temperature the propylene oxide vaporized so that the oxide penetrated the cotton plug thus fumigating the media. The container remained sealed for twelve hours. At the end of this period it was opened to allow exhaustion of the gas. Twenty-four hours later, after all trace of the gas had been removed, the cultures were inoculated.

Inoculation was effected by means of a standard inoculating needle during which a small amount of surface mycelium was scraped from two- to three-week old agar slants and transferred under aseptic conditions to the culture medium. These transfers were made as uniform as possible.

Cultures in Erlenmeyer and Kolle flasks were maintained in the laboratory at room temperature (22–26°). McCrea (2) has found that *Claviceps purpurea* grows satisfactorily over a wide temperature range, i. e., 18–30°.

Since aeration-agitation has been known to be influential in the metabolic activities of mold cultures from all aspects and this has been confirmed in the case of *Claviceps* culture by other workers (6, 7), growth was cultured either continuously or by intermittent shaking in Erlenmeyer flasks by the use of a mechanical reciprocating shaker and a Cutler-Hammer rotary shaker. The Kolle culture flasks or standing flasks were placed on open laboratory shelves, for observation.

Wickerham Formula (9, 10) and a yeast extract-peptone formula (6) were selected for control purposes so that the growth produced by the homogenated tissues could be compared with that supported by a semisynthetic medium. The Wickerham formula consisted of 500 mg. yeast carbon base, 7 mg. maltose, and 400 mg. ammonium sulfate per 100 ml. distilled water. All cultures in each experiment were inoculated on the same day with the Minnesota (Stakman) strain of *Claviceps*.

Growth on Rye Mash.—Mashes of the three stages of inflorescence which had been kept frozen in a freezing compartment were placed in distilled water to produce a thick green suspension. One-half of these were sterilized by propylene oxide sterilization described previously and one-half were autoclaved. Those sterilized by gas remained a green color whereas those which had been autoclaved became brownish green due to the effects of heating. These cultures were then inoculated and one group shaken; the second allowed to stand in Kolle flasks. A similar inoculation was prepared during which Wickerham's formula was employed as the control.

In a ten-day period no growth was observed to appear on any material prepared with the rye mash of the immature inflorescences. However, at the end of eighteen days of growth, several brown, gel-like fungus pellicles were found to develop on mashes prepared from homogenates of the mature

Fig. 1.—Mycelial pellicles and gel-like masses of *Claviceps purpurea* growing on a mash suspension prepared from mature (fruiting) rye inflorescences. Growth period—18 days.

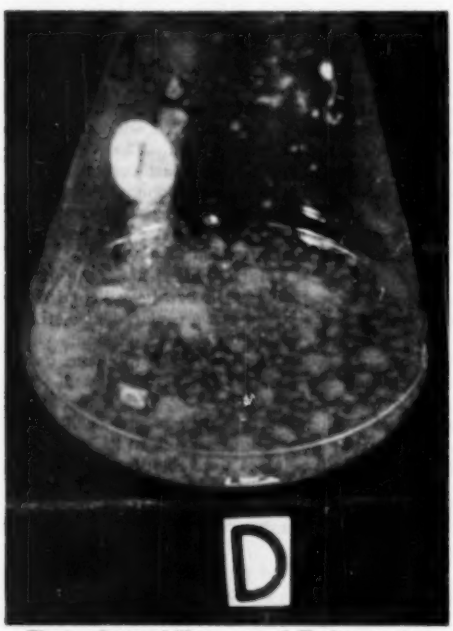

Fig. 2.—Oatmeal-like masses of *Claviceps purpurea* growing on a liquid filtrate of immature rye inflorescences. Growth period—12 days.

inflorescence (fruiting stage) (Fig. 1). Within a ten-day growth period all cultures prepared with Wickerham control formula produced good growth. Many small, dull, opaque sizes formed in shake flasks. A gray-white gelatinous pellicle about 0.25 cm. covered the surface area in the still (Kolle) cultures.

Microscopic examination of those homogenates (mature inflorescence) which exhibited growth in an eighteen-day period showed numerous *Claviceps* hyphae with few cross walls and many conidiospores. The mycelium conformed to known *Claviceps* mycelia grown in this laboratory and reported by others (2, 5–7). However, to ascertain more definitely whether or not it was *Claviceps* growth, transfers of the material were placed on subcultures employing the media of Schwarting and Hiner (11) with a liquid yeast extract-peptone formula and shaking. Numerous gray-white globular pellicles were formed on this medium and in all respects the mycelia tissue resembled that of authentic *Claviceps* growth.

It appeared possible that the chlorophyll present in the media might have inhibitory action on culture growth of the fungus as this effect has been reported in the case of other fungi (12). Therefore, chlorophyll was removed from the homogenates by shaking with an excess of acetone for twenty-four hours until no green color remained. Cultures were inoculated upon complete removal of the acetone and these were then developed as previously. No significant growth ensued on any of these cultures.

In order to determine what influence heat would have on the inflorescence materials, such as that necessary to completely dry it prior to its use in cultures freshly collected inflorescences were dried at 46° for twenty-four hours and powdered in a laboratory Wiley mill. This material was then made into a water suspension consisting of 10 Gm. of powdered rye and enough distilled water to make a suspension volume of 100 ml. in shake flasks and 6 Gm./60 ml. in still (Kolle) flasks. It was then sterilized, inoculated, and placed into shaking and Kolle flasks for growth. At the end of eighteen days, growth was observed only on material prepared from the mature inflorescence (fruiting) stage which had been autoclaved. This consisted of white woolly patches which upon microscopical examination possessed characteristics similar to those previously noted. Fewer conidiospores, however, appeared to be present. This growth was subcultured on a liquid yeast extract-peptone medium, for a ten-day period and good growth was produced. This confirmed its *Claviceps* characteristics.

The three inflorescence stages were then collected at appropriate intervals and each processed immediately as fresh material for culture purposes after cutting the rye heads in the field. It was felt that factors necessary for more adequate growth than heretofore shown had been inhibited under the conditions of freezing and/or drying such as had been effected during the previous harvesting technique. The growth experiment was thus repeated with fresh material using otherwise identical methods as previously described. Growth appeared within an eighteen-day period in both shake and still cultures of the freshly homogenated mature inflorescence (fruiting) stage, but only that which

had been previously autoclaved. It did not appear on the other cultures. It was concluded that mature inflorescence material sterilized by propylene oxide failed to produce growth because of the incomplete removal of the gas prior to inoculation of the cultures. Microscopically, the growth on the autoclaved mature inflorescence culture was characterized by the presence of brown gel-like pellicles intermixed with gray-white mycelial strands. Typical *Claviceps* hyphae with few cross walls were noted under the microscope. This material was subcultured on semisynthetic media as previously described and its authenticity as *Claviceps* growth confirmed.

Growth on Liquid Phase of Rye Homogenates.—The strained homogenated material separated from the mash, called the liquid phase of the three stages of the rye inflorescence, was then placed into culture flasks provided for shaking and still culture (eight flasks for each experimental condition). Material from fresh, frozen, and dried rye heads was employed. These cultures were then arranged as for the mash cultures. One-half of these were sterilized with propylene oxide and one-half were autoclaved. Those sterilized by gas remained green in color whereas those which were autoclaved became brownish amber with a flocculent precipitate floating in the medium. This was due to the effects of heating, and the solid particles were retained in the culture. These cultures were then inoculated and one group shaken, the second allowed to stand in Kolle flasks.

Within twelve days all still cultures which had been sterilized previously by gas exhibited growth. This occurred as gray-brown gel-like fungus pellicles with white woolly patches. However, those shake flasks with strained homogenate liquid from the freshly processed mature (fruiting) rye inflorescence produced the best growth. This appeared as thick grayish white, oatmeal-like *Claviceps* masses. The microscopic examination of all growth confirmed its similarity to that of known *Claviceps*. Many more conidiospores were noted, however, in the material separated from still (Kolle) cultures. Transfers of growth from this liquid phase were made to a semisynthetic medium as from the growth experiment previously described for confirming the authenticity of mash culture growth. Identical *Claviceps* mycelial development resulted.

In order to test the factor of chlorophyll inhibition on growth in these cultures, a duplicate batch with the chlorophyll removed was inoculated at the same approximate time. Removal of the chlorophyll was effected by ether extraction instead of acetone as employed for mash culture media so that the resultant chlorophyll-ether liquid layer could be decanted more conveniently. The separated aqueous layer was asperated with suction for twenty-four hours to insure the complete separation of the ether. The chlorophylless liquid employed for culture purposes was a brown-amber liquid. No growth was noted in an eighteen-day period on any of these cultures. From these results and those noted when using chlorophyll-less rye mashes, it was concluded that a substance or substances associated with chlorophyll are essential for *Claviceps* growth under these conditions.

As the result of this test, an attempt was then made to culture *Claviceps* growth on the decanted liquid of the centrifuged liquid phase (green suspension) prepared from all three stages of the inflorescence. The filtrate from centrifuging retained some of the green chlorophyll pigment, but was devoid of plastids and other cellular elements that were present in the original liquid. These cultures were sterilized by the gas and autoclave methods and the culture liquids were yellow-green and brownish green, respectively, indicating some pigmentation. Solid particles separated in the autoclaved material as previously noted.

Good *Claviceps* growth was produced by the inoculum in both shake and still flasks within five to ten days (Fig. 2). In several cases many white and globular pellicles were found on the liquid surface and in other instances, particularly the still cultures, the pellicles covered the entire surface. Microscopic examination of these revealed the presence of hyphae and many conidiospores. Subculture checks using this material on semisynthetic yeast extract-peptone media also resulted in positive *Claviceps* growth.

A summary of the comparative growth of rye mash and liquid phases is shown in Table I.

Inoculation of Flowering Rye.—In order to test the viability of *Claviceps* growth previously achieved on rye mash suspensions and liquid filtrates an inoculum culture consisting of the suspension and/or liquid phase was employed for inoculating flowering rye plants. These were grown in an experimental plot in the greenhouse under conditions of high humidity and temperature (23-26°). Flowering heads were dipped into the *Claviceps* homogenate culture so that complete saturation was effected. A total of five such inoculations were made in a ten-day interval. Within six days a majority of the flowering heads were in the *Claviceps* honeydew stage. In fourteen days sclerotia were in complete development in the majority of the infected rye flowers (Fig. 3). The ergots were compared with known ergots from commercial samples and found to be identical. This confirmed the viable and true nature of the mycelial growth as described.

TABLE I.—CLAVICEPS GROWTH ON VARIOUS CULTURES OF RYE (SECALE CEREALE) INFLORESCENCE HOMOGENATES

Preparation	Stage of Inflorescence	No. Cultures	Days of Growth	Growth	
				Chlorophyll Present	Chlorophyll Absent
Mash	Nonflowering	16	18	−	−
	Flowering	16	18	−	−
	Fruiting	16	18	+	−
Liquid phase	Nonflowering	16	12	+	−
	Flowering	16	12	+	−
	Fruiting	16	12	++	−

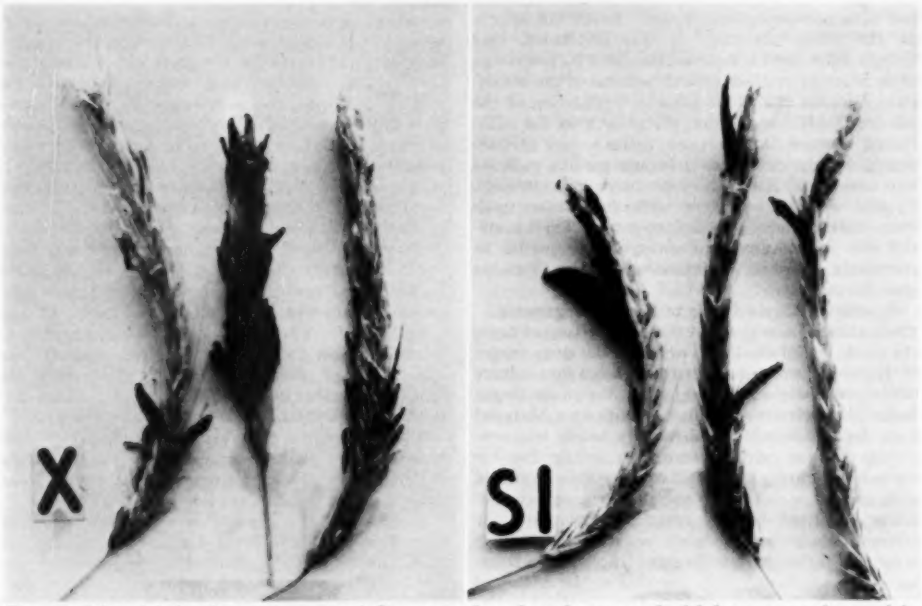

Fig. 3.—Sclerotial development on rye inflorescence from inocula prepared with homogenated materials. *X*—inoculum from rye mash cultures; *SI*—inoculum from liquid filtrate cultures.

SUMMARY AND CONCLUSIONS

1. A qualitative comparison of *Claviceps purpurea* mycelia growth on various substrates prepared from three stages of rye inflorescence has been made.

2. Good growth was obtained on all liquid filtrates of each of these stages. However, in the case of rye mash suspensions, growth occurred only on culture material prepared from mature (fruiting) inflorescences. In no instances were sclerotial tissues observed.

3. There was evidence during a comparison of cultures with and without the presence of chlorophyll that this substance or substances associated with it under the conditions of extraction possess factors which are necessary for *Claviceps* growth.

4. In preparing materials for cultures no differences were noted between the use of inflorescences which had been previously frozen, dried, or employed in the fresh condition.

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The Culture of *Claviceps purpurea**

II. Preliminary Metabolism Studies

By A. G. PAUL,† W. J. KELLEHER,† and A. E. SCHWARTING

Various compounds representing structural moieties of lysergic acid were added to submerged cultures and to replacement cultures of *Claviceps*. Qualitative studies for probable alkaloid synthesis and quantitative utilization of some of these compounds are reported. The effect on growth and a new method for the detection of alkaloids on paper chromatographs are demonstrated.

ATTEMPTS AT PRODUCING the alkaloids of ergot in saprophytic culture date back to Bonns (1) who succeeded in growing *Claviceps purpurea* on a variety of complex media. Extracts of the fungus growth failed to show any physiological activity. Since then various workers (2–8) have reported success in producing the alkaloids. However, either because of cumbersome techniques or insignificant yields, their methods have not been employed commercially.

An understanding of the mechanisms by which the ergot alkaloids are produced, and consequently success in the production of them, might well be demonstrated by attempting to establish their pathway of synthesis.

The structure common to all of the physiologically active alkaloids is *d*-lysergic acid and in this structure can be seen the semblance of anthranilic acid, indole, tryptophan, amino-N-methyltryptophan, nicotinic acid, quinolinic acid, pyridoxine, and trigonelline. The compounds represented by these moieties, with the exception of amino-N-methyltryptophan and trigonelline, are known to be commonly synthesized or utilized by a wide variety of microorganisms.

While the appearance of these compounds does not necessarily indicate that they participate in the biosynthesis of lysergic acid, it would seem justified, considering that many do play a part in the metabolism of several microorganisms, to attempt to show whether or not they are utilized by *Claviceps* and if so, whether or not this utilization has any effect on alkaloid production by the organism.

EXPERIMENTAL

Organism.—Recent isolates of strains from the sclerotia of domestic rye ergot were used in all studies.

Growth.—The medium used was that shown to yield maximum growth (9)—2.0% mannitol and 1.0% casein hydrolysate in a mineral solution. One hundred milliliters of the medium in a 500-ml. Erlenmeyer flask was sterilized in an autoclave at 121° for fifteen minutes. Such a flask was inoculated from a slant culture of the organism and incubated at 21–23° on a reciprocal shaker until sufficient growth was evidenced (five to seven days). All flasks for a particular experiment were inoculated from such a stock flask by means of a transfer cup, perforated at the base and holding the volume of approximately 0.8 ml. In turn, these flasks were incubated on a reciprocal shaker for seventy-two hours.

At the end of this incubation period, the flasks were treated as follows:

(*a*) Filter sterilized solutions of indole (10.0 mg.), indole (10.0 mg.) and DL-serine (10.0 mg.), L-tryptophan (50.0 mg.), *d*-amino-N-methyltryptophan (50.0 mg.), anthranilic acid (50.0 mg.), quinolinic acid (10.0 mg.), nicotinic acid (10.0 mg.), indole (10.0 mg.) and quinolinic acid (10.0 mg.), indole (10.0 mg.) and nicotinic acid (10.0 mg.), indole (10.0 mg.) and pyridoxine hydrochloride (10.0 mg.), and indole (10.0 mg.) and trigonelline bisulfate (10.0 mg.) were added to the cultures and the cultures incubated for twenty-four hours.

(*b*) The cultures were centrifuged aseptically, the mycelia washed and transferred to flasks containing the filter-sterilized test compound in 100 ml. of isotonic NaCl solution. These replacement cultures were then incubated for twenty-four hours on a reciprocal shaker. Studies by this method were limited to indole (5.0 mg.), indole (5.0 mg.) and DL-serine (100.0 mg.), L-tryptophan (9.0 mg.), and *d*-amino-N-methyltryptophan (9.0 mg.).

Qualitative Analysis for Alkaloids.—Those cultures to be analyzed for alkaloids were frozen in a dry ice-alcohol mixture and placed in a Stokes freeze-drier until complete desiccation was effected.

The dried products were extracted by the N. F. (10) procedure for the assay for total alkaloids of ergot. The chloroform extracts were reduced to a small volume and chromatographed (11). The alkaloids fluoresce blue and react with *p*-dimethylaminobenzaldehyde in acid solution to give a blue color. It was established that 2% of the aldehyde in HCl was sensitive to a concentration of ergonovine on the order of 20 μg. A reagent found to be sensitive to 5 μg. of ergonovine was also used (12). The chromatograms were sprayed with a 1% solution of cinnamic aldehyde in methanol, dried, and exposed to HCl vapor. Ergonovine gives a pink color.

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Analysis for Test Compounds and Products of Dissimilation.—These analyses were confined to replacement cultures. After the twenty-four hour incubation, the mycelium was filtered off and the filtrates reserved for both qualitative and quantitative analyses.

Paper partition chromatography was used to qualitatively identify the compounds. Portions of 0.1 ml. of the filtrates were spotted on sheets of Whatman No. 1 filter paper, 18.25 x 22.5 in., and the chromatogram developed with the organic phase of a butanol-glacial acetic acid-distilled water mixture (4:1:5) by the ascending technique. Spraying with 2% *p*-dimethylaminobenzaldehyde in HCl was used to detect the zones. With this reagent indole gives a pink color; tryptophan and amino-N-methyltryptophan give a blue color.

By this method, indole has an R_f value of 0.98, tryptophan, 0.56, and amino-N-methyltryptophan, 0.76.

Indole was determined quantitatively by the method of Wood, *et al.* (13), using a Beckman spectrophotometer at a wave length of 540 mμ. Tryptophan and amino-N-methyltryptophan were both assayed by the method of Steers and Sevag (14), with a Beckman spectrophotometer at a wave length of 560 mμ.

Growth Weight Studies.—To determine whether or not the compounds in replacement culture had any effect on the growth weight, cultures were grown in a medium differing from the growth medium employed previously in that 1.0% Bacto casamino acids was used in place of 1.0% casein hydrolysate. This organic nitrogen source is free of tryptophan.

Indole, L-tryptophan or *d*-amino-N-methyltryptophan were added directly to this medium, the media were filter sterilized, and then inoculated with 1.0 ml. of a previously blended stock flask culture. These flasks were incubated for ninety-six hours on a reciprocal shaker.

At the end of this time, the mycelium from each flask was filtered and dried to a constant weight.

The weights were analyzed statistically[1] by computing the "Student *t*" (15) and then obtaining the probability point from statistical tables (16) with reference to the number of degrees of freedom.

Filtrates were analyzed quantitatively for indole, tryptophan, and amino-N-methyltryptophan by the methods noted earlier.

RESULTS

Qualitative Analysis for Alkaloids.—Chromatographic analysis failed to indicate the presence of any of the ergot alkaloids in the extract of the dried mycelia-media complexes of cultures in which the test compounds had been added directly to the media after seventy-two hours of growth, nor could they be detected in the extracts of the replacement cultures.

However, compounds not present in the controls and not accounted for by the added test compounds were noted in several instances.

Analysis of the Filtrates of Replacement Cultures.—Chromatograms of the filtrates of replacement cultures to which indole and indole plus serine had been added indicated that no indole remained after twenty-four hours. The presence of a compound giving an R_f value of 0.56 and which did not separate from known L-tryptophan was observed. This compound was not present in controls.

Quantitative analysis of these filtrates substantiated the fact that no indole remained and that approximately 25 μg. of tryptophan per ml. of solution was present in the filtrates of cultures to which indole alone had been added and 24 μg. per ml. in filtrates of cultures to which both indole and DL-serine had been added.

Qualitative analysis of the filtrates of cultures to which known L-tryptophan had been added showed that some tryptophan remained after twenty-four hours. Quantitative tests indicated that of the 90 μg. per ml. of solution added, 55 μg. was utilized.

It was demonstrated that amino-N-methyltryptophan was utilized to the extent of approximately 22 μg. per ml. of solution.

Growth Weight Studies.—The statistical analysis of the mycelial weights is summarized in Table I.

Quantitative analysis of the filtrates showed that indole was utilized completely in ninety-six hours and that approximately 24 μg. of tryptophan was produced from 50 μg. of indole, per ml. of solution, and still remained.

The amount of added tryptophan (90 μg. per ml.) utilized in ninety-six hours was approximately 30 μg. per ml.; the amount of amino-N-methyltryptophan utilized was negligible.

DISCUSSION AND CONCLUSIONS

Under the conditions studied, various compounds representing several structural moieties of lysergic acid apparently do not influence the production of the ergot alkaloids by *Claviceps purpurea*.

It was shown that indole, at a concentration of 50 μg. per ml. of solution and in the absence of other organic material, was utilized completely and that approximately 25 μg. of tryptophan per ml. of solution was produced from it and still remained at the end of twenty-four hours. Over this twenty-four hour period, the effect of serine was not demonstrable.

Added L-tryptophan (90 μg. per ml.) was utilized to the extent of 55 μg. in twenty-four hours; added amino-N-methyltryptophan (90 μg. per ml.) was utilized to the extent of 22 μg.

The data seem to indicate that indole is converted almost entirely to tryptophan by *Claviceps* and the tryptophan in turn is utilized to yield an undetermined compound or compounds.

Amino-N-methyltryptophan is utilized to some extent by the organism; whether it is part of a biosynthetic pathway or whether it merely substitutes for tryptophan has not been established.

In the presence of other organic materials, the amounts of tryptophan and amino-N-methyltryptophan utilized in a given time are decreased.

Statistical analysis of the effects of indole, tryptophan, and amino-N-methyltryptophan on the growth weight of *Claviceps* indicates that tryptophan, under the conditions set forth, significantly inhibits the growth of the organism. The same cannot be concluded for indole and for amino-N-methyltryptophan.

[1] The statistical analysis was performed by Miss Lois Decker of the University of Connecticut, Storrs, Conn.

TABLE I.—STATISTICAL ANALYSIS OF GROWTH WEIGHTS

	Number of Flasks	Mean Dried Weight (Mg.)	t	Probability
Control	8	946.9	1.333	<0.3
Indole	9	871.0		
Control	8	946.9	4.115	<0.001
Tryptophan	10	727.8		
Control	8	946.9	0.3587	<0.8
Amino-N-methyl-tryptophan	10	968.6		
Indole	9	871.0	3.192	<0.01
Tryptophan	10	727.8		
Indole	9	871.0	1.864	<0.1
Amino-N-methyl-tryptophan	10	968.6		
Tryptophan	10	727.8	4.912	<0.001
Amino-N-methyl-tryptophan	10	968.6		

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The Culture of *Claviceps purpurea**

III. Tryptophan Metabolism

By V. E. TYLER, Jr.† and A. E. SCHWARTING

Claviceps purpurea **was produced in submerged culture in a medium of known chemical composition and the mycelium tested for its ability to synthesize tryptophan and related compounds in replacement culture. Additions of indole resulted in its utilization with subsequent tryptophan production. Added serine increased the rapidity of this reaction, but the requirement for serine was not absolute. Anthranilic acid and shikimic acid failed to serve as tryptophan precursors. Tryptophan disappeared from the replacement cultures in a relatively short time, but lysergic acid was not formed. Nicotinic acid was identified as a product of tryptophan utilization.**

THE SYNTHESIS and utilization of tryptophan in *Claviceps purpurea* (Fries) Tulasne are especially important since the medicinally useful ergot alkaloids are all derivatives of lysergic acid, a compound which possesses a biogenetic relationship to tryptophan (1). It has been established (2) that *Claviceps*, although capable of mycelial development in submerged culture, fails to produce appreciable quantities of lysergic acid or its derivatives when cultivated in this manner. However, the organism was found to be capable of the utilization of indole from a replacement culture (3), and tryptophan formation was shown to correlate with indole disappearance (4).

Only in recent years have some of the biosynthetic pathways, by which tryptophan is formed, been elucidated. Using mutant strains of *Neuro-*

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spora crassa, the pathway anthranilic acid → indole (+ serine) → tryptophan was established by Tatum, Bonner, and Beadle (5–7). Similar pathways have been reported to exist in *Salmonella typhosa*, *Escherichia coli* (8) and *Lactobacillus arabinosus* (9). Preparation of a cell-free enzyme system from *Neurospora sitophila*, capable of producing tryptophan from indole and serine was reported by Umbreit, Wood, and Gunsalus (10). The name, tryptophan desmolase, has been conferred upon the enzyme responsible for this reaction (11). Yanofsky (12) has investigated the properties of a partially purified tryptophan desmolase preparation obtained from the wild type *Neurospora*.

Davis (13) produced evidence which established the importance of shikimic acid and 5-dehydroshikimic acid in aromatic (tryptophan) biosynthesis by *Escherichia coli*.

Beadle, Mitchell, and Nyc (14, 15), using *Neurospora*, showed that nicotinic acid was normally formed from tryptophan in that organism, and that kynurenine and 3-hydroxyanthranilic acid were successive intermediates in this reaction.

This investigation was undertaken to determine which of these various reactions of tryptophan metabolism could be shown to occur in *Claviceps* and their relationship, if any, to lysergic acid biosynthesis.

EXPERIMENTAL

Production of Mycelium.—The strain of *Claviceps* used in this study was isolated from a sclerotium of domestic barley ergot and maintained in pure culture. In order to eliminate constituents of unknown identity from the culture medium, the organism was subjected to a process of physiological adaptation, until after repeated transfer it was capable of prolific mycelial development in a medium of definite chemical composition.

The mycelium used throughout the investigation was produced in submerged culture at room temperature in the following medium:

NH_4NO_3	1.0	Gm.
KH_2PO_4	2.0	Gm.
$MgSO_4 \cdot 7H_2O$	0.5	Gm.
$CaCl_2$	0.1	Gm.
$FeSO_4 \cdot 7H_2O$	0.1	Gm.
$Na_2B_4O_7 \cdot 10H_2O$	0.088	mg.
$CuSO_4 \cdot 5H_2O$	0.392	mg.
$MnCl_2 \cdot 4H_2O$	0.072	mg.
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.037	mg.
$ZnSO_4 \cdot 7H_2O$	8.81	mg.
Mannitol	20.0	Gm.
DL-Alanine	1.0	Gm.
L-Asparagine	1.0	Gm.
L-Aspartic acid	1.0	Gm.
L-Glutamic acid	1.0	Gm.
L-Leucine	1.0	Gm.
DL-Valine	1.0	Gm.
Distilled water, to make 1,000 ml.		

Replacement Culture Technique.—A standardized procedure was developed and adopted for all studies of biosynthesis. A quantity of the compound(s) to be tested for utilization was placed in a calibrated test tube with 2 ml. of distilled water. An 0.8-ml. volume (ca. 50 mg. dry weight) of mature, washed mycelium was transferred to each tube. Sufficient 1/15 *M* phosphate buffer (16) at *p*H 7.5 was added to produce a total volume of 5 ml. in each tube. The tubes were then loosely stoppered and placed on a reciprocal shaking machine at room temperature for the specified period of time. Three replications of each sample were usually prepared.

When the reaction mixtures which contained added indole were assayed for that substance, a 10-ml. quantity of toluene was added to each tube and shaken thoroughly to remove unutilized indole and stop the reaction. Aliquots of the filtered toluene layer were assayed for indole by the method of Wood, Gunsalus, and Umbreit (17). After filtering, the lower aqueous layer was used for the determination of tryptophan which was carried out essentially as described by Nason, Kaplan, and Colowick (18). If indole was known to be absent, the mycelium was filtered from the mixture and the filtrate assayed directly for tryptophan. Determinations of anthranilic acid also were carried out directly on the filtrate, with the use of Rhuland and Bard's (19) modification of the procedure of Bratton and Marshall (20). In all of the assay procedures the optical density of the solutions was determined with a Beckman, model D. U. spectrophotometer.

Utilization of Indole and Production of Tryptophan.—To determine the quantitative aspects of indole uptake and tryptophan synthesis by washed *Claviceps* mycelium, a series of tubes was prepared as previously described, each of which contained 95.5 μg. of indole. After shaking for periods of one, two, four, and eight hours, replicate tubes were harvested and assayed for indole and tryptophan. The effect of added serine was also studied by treating a set of tubes as previously described, with the exception that 2 mg. of DL-serine was added to each tube in addition to the 95.5 μg. of indole. The results of this series of assays are expressed on both a weight and a molar basis in Table I.

From these data it was apparent that added serine had considerable effect upon the rapidity with which indole utilization and subsequent tryptophan formation occurred. The requirement of added serine for tryptophan formation was by no means absolute, and since no serine was added to the original culture medium, this was interpreted as signifying that the organism was capable of limited serine synthesis.

To verify that the product formed by indole and serine coupling was actually tryptophan, a concentrated extract of the replacement culture was subjected to paper-partition chromatography. This extract was prepared by freeze-drying an indole-serine replacement culture which had been shaken with mycelium for eight hours and then filtered. The dried residue was redissolved in a minimum quantity of warm water.

Portions of this solution spotted on strips or sheets of Whatman No. 1 filter paper were chromatographed with two different wash liquids. A mixture of *n*-butanol-acetic acid-water (4:1:5) in which the R_f values of the ergot alkaloids have been determined

TABLE I.—RELATIONSHIP BETWEEN INDOLE UTILIZATION AND TRYPTOPHAN FORMATION WITH AND WITHOUT ADDED SERINE

Time Shaken (Hours)	Indole Utilized		Tryptophan Formed	
	μg.	μM × 10³	μg.	μM × 10³
	Indole Added 95.5 μg.			
1	9.2 ± 2.7	7.3 ± 2.3	18.8 ± 1.3	9.2 ± 0.6
2	24.5 ± 3.0	20.9 ± 2.6	31.2 ± 1.3	15.3 ± 0.7
4	41.2 ± 3.7	35.2 ± 3.2	45.8 ± 5.2	22.4 ± 2.6
8	81.8 ± 1.7	69.8 ± 1.4	108.5 ± 8.5	53.1 ± 4.7
	Indole (95.5 μg.) and DL-Serine (2 mg.) Added			
1	15.5 ± 3.0	13.2 ± 2.6	20.5 ± 3.0	10.0 ± 1.5
2	36.8 ± 2.3	31.4 ± 2.0	49.8 ± 2.3	24.4 ± 1.1
4	90.2 ± 0.7	77.0 ± 0.6	88.3 ± 7.8	43.2 ± 3.9
8	95.5 ± 0.0	81.5 ± 0.0	146.0 ± 8.0	71.5 ± 3.9

(21), and a methanol-*n*-butanol-benzene-water (4:2:2:2) mixture used by Mason and Berg (22) to separate and identify the products of tryptophan metabolism were the solvent systems used. Ascending technique was used throughout; the time of formation approximated forty-eight hours in the former system and ten to twelve hours in the latter. The spots were localized on the sheets by spraying with ninhydrin solution (0.4% in water-saturated butanol) or with 2% *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid. By comparison with control substances of known identity, also spotted in a concentrated buffer solution, tryptophan was identified as the product formed from indole and serine by *Claviceps*.

Anthranilic acid was also identified as a by-product of the indole-serine reaction, although only small amounts were formed. Anthranilic acid has been isolated by Partridge, Bonner, and Yanofsky (23) from a quinolinic acid-producing *Neurospora* mutant grown on N^{15}-containing indole.

Utilization of Anthranilic Acid.—Anthranilic acid was tested to see if it also might serve as a precursor to tryptophan in *Claviceps*. Using the replacement culture technique previously described, a series of tubes, each containing 104 μg. of anthranilic acid and 2 mg. of DL-serine, was prepared. These were shaken for eight hours at room temperature and the contents assayed for remaining anthranilic acid. Another series of tubes was prepared, each containing 10 mg. of D-glucose in addition to the indicated amounts of anthranilic acid and serine. Glucose has been shown to be an absolute requirement for anthranilic acid utilization by *Lactobacillus arabinosus* (19). The results of these trials are summarized in Table II.

Considerable anthranilic acid was utilized in the eight-hour time interval under the conditions described. The uptake was approximately doubled in the same time interval when glucose was added. In order to determine if either indole or tryptophan was formed from the anthranilic acid utilized, quantities from the tubes with anthranilic acid, serine, and glucose added were spotted on strips of Whatman No. 1 filter paper and chromatographed with the *n*-butanol-acetic acid-water wash liquid. No tryptophan or indole could be detected although the remaining anthranilic acid was readily identified.

Utilization of Shikimic Acid.—Three series of replacement culture tubes containing 100 μg. of shikimic acid, 100 μg. of shikimic acid and 2 mg. of DL-serine, and 100 μg. of shikimic acid plus 10 mg. of D-glucose, respectively, were prepared. After shaking for eight hours at room temperature the tubes were extracted with toluene and assayed for indole and tryptophan. Neither of these compounds was detected in the replacement cultures under the conditions described.

TABLE II.—ANTHRANILIC ACID UTILIZATION

Time Shaken (Hours)	No. Samples	Anthranilic Acid (Av.) Recovered (μg.)	Anthranilic Acid (Av.) Utilized (μg.)
Anthranilic Acid (104 μg.) and DL-Serine (2 mg.) Added			
8	4	91.0 ± 3.0	13.0 ± 3.0
Anthranilic Acid (104 μg.), DL-Serine (2 mg.) and D-Glucose (10 mg.) Added			
8	3	76.7 ± 5.3	27.3 ± 5.3

Utilization of Tryptophan.—Although *Claviceps* was found to be capable of synthesizing tryptophan from indole and serine, it was found that when tryptophan was added to the replacement culture, it eventually disappeared. To investigate this phenomenon and to obtain some knowledge of the conditions which might influence tryptophan disappearance, eight series of replacement cultures were prepared, consisting of the following additions:

1. Tryptophan (101 μg.) and water.
2. Tryptophan (101 μg.) and buffer.

TABLE III.—TRYPTOPHAN UTILIZATION

Time Shaken (Hours)	No. Samples	Tryptophan (Av.) Added (μg.)	Tryptophan (Av.) Recovered (μg.)	Tryptophan (Av.) Utilized (μg.)
Water				
8	3	101.0	0	101.0
Buffer				
8	3	101.0	0	101.0
DL-Serine (2 mg.)—Water				
8	3	101.0	33.7 ± 4.7	67.3 ± 4.7
DL-Serine (2 mg.)—Buffer				
8	3	101.0	33.7 ± 4.3	67.3 ± 4.3

TABLE IV.—INDOLE UTILIZATION AND TRYPTOPHAN FORMATION

Time Shaken (Hours)	No. Samples	Indole (Av.) Recovered (μg.)	Indole (Av.) Utilized (μg.)	Tryptophan (Av.) Recovered (μg.)
		Indole (89 μg.)—DL-Serine (2 mg.)—Water		
8	3	0	89.0	83.2 ± 6.2
		Indole (89 μg.)—DL-Serine (2 mg.)—Buffer		
8	3	0	89.0	62.3 ± 5.7
		Indole (89 μg.)—Tryptophan (101 μg.)—Buffer		
8	3	16.7 ± 2.7	72.3 ± 2.7	77.7 ± 7.7
		Indole (89 μg.)—Tryptophan (101 μg.)—DL-Serine (2 mg.)—Buffer		
8	2	0	89.0	128.5 ± 3.5

3. Tryptophan (101 μg.) plus DL-serine (2 mg.) and water.
4. Tryptophan (101 μg.) plus DL-serine (2 mg.) and buffer.
5. Indole (89 μg.) plus DL-serine (2 mg.) and water.
6. Indole (89 μg.) plus DL-serine (2 mg.) and buffer.
7. Indole (89 μg.) plus tryptophan (101 μg.) and buffer.
8. Indole (89 μg.) plus tryptophan (101 μg.) plus DL-serine (2 mg.) and buffer.

As before, the constituents were added in 2 ml. of distilled water, washed mycelium was added, and water or 0.15 *M* phosphate buffer at *p*H 7.5 was used to bring the volume to 5 ml. After shaking for eight hours at room temperature, tubes of the first four series were assayed for remaining tryptophan, those of the last four were shaken out with toluene and assayed for both indole and tryptophan. The results of this investigation are summarized in Tables III and IV.

Quantitative differences in the amount of tryptophan formed were obtained with different lots of mycelium of approximately the same physiological age. The results reported in Tables III and IV were obtained from a single lot of mycelium. It was apparent that, for this particular culture, the eight-hour shaking period was somewhat too long since the yield of tryptophan was small even though all of the indole had been utilized. It was also assumed that tryptophan was originally produced in nearly theoretical amount, but was subsequently metabolized to other products.

Identification of Nicotinic Acid.—It was suspected that one of the products of tryptophan utilization might be nicotinic acid. Therefore, a method was sought for the detection and identification of that compound in micro quantities. Munier (24) documented the R_f values given by nicotinic acid when subjected to paper partition chromatography in various solvent systems. His Ba 30 wash liquid, consisting of *n*-butanol-glacial acetic acid-water (100:30:88) was used. According to Munier, nicotinic acid gave an R_f value of 0.74 in this system.

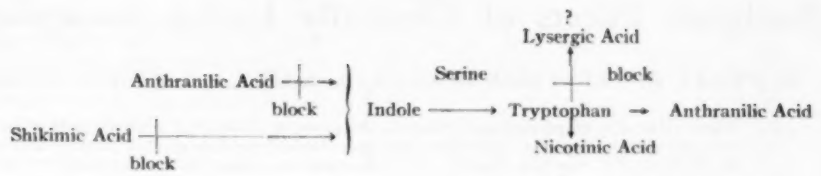
Mueller and Fox (25) were able to detect microgram quantities of nicotinic acid in solution by using an ammonia buffer composed of 0.1 *M* ammonium hydroxide, 0.5 *M* dipotassium phosphate, and 2.0 *M* ammonium chloride, in combination with 10% aqueous cyanogen bromide solution. With nicotinic acid a yellow color was formed which reached maximum intensity in two to two and one-half minutes and then faded rapidly.

Attempts to render visible a nicotinic acid spot on paper using these reagents in the form of a spray were unsuccessful. However, when the nicotinic acid-containing spot was cut out and placed in a small test tube together with 0.5 ml. of ammonia buffer and 1 ml. of the cyanogen bromide solution, a clear yellow color was formed in the solution. This procedure was adopted as a sensitive means of localizing nicotinic acid on the paper strips.

In order to determine if nicotinic acid was a product of tryptophan utilization, two replacement cultures were prepared in 125-ml. Erlenmeyer flasks. The first contained approximately 1.2 mg. of tryptophan in 12 ml. of water, to which was added washed mycelium (*ca.* 0.6 Gm. dry weight) and sufficient distilled water to adjust the volume to 60 ml. The second flask was prepared in the same manner, with the omission of tryptophan. Both flasks were shaken for eight hours at room temperature, the mycelium was removed by filtration, and the filtrates were freeze-dried. The dried residues were taken up in distilled water and filtered. Colorimetric and chromatographic procedures indicated that only a minute quantity of tryptophan remained in the first flask.

Subsequently, portions of the solutions from both flasks were spotted on Whatman No. 1 filter paper and subjected to ascending formation using Munier's Ba 30 wash liquid. Control strips containing quantities of known nicotinic acid were chromatographed simultaneously.

After drying, the strips were cut into 30-mm. sections, each of which was tested for nicotinic acid by the method just described. Four standard strips showed that all of the nicotinic acid was localized in an area between R_f 0.72 and 0.79 (average 0.755), and no positive test could be obtained with sections of the strips above or below these positions. Sections cut at R_f 0.72–0.79 from six different strips, spotted with quantities of the solution from the tryptophan-added flask, all gave strong positive reactions for nicotinic acid. No indication of a positive reaction was obtained with sections cut from any other position on these strips. Sections from strips spotted with equivalent quantities of the control flask solution (no tryptophan added) failed to give any yellow color between R_f 0.72 and 0.79.



DISCUSSION

Indole was not accumulated by saprophytically-cultured *Claviceps* mycelium. Additions of that compound to replacement cultures of the organism resulted in the utilization of indole and the production of tryptophan. Indole was thus shown to function as a tryptophan precursor.

Although additions of serine were shown to increase the rapidity of indole uptake and tryptophan formation, the requirement for serine was a relative one. It was concluded, therefore, that the organism was capable of synthesizing serine in limited amounts, since that amino acid was not added to the culture medium. The synthesis of tryptophan by the condensation of indole and serine corresponds to that reaction which exists in certain *Neurospora* mutants and which has been postulated to occur in certain bacteria.

A second compound formed from indole in addition to tryptophan proved to be anthranilic acid. The mechanism by which this compound was formed is obscure. The formation of anthranilic acid from indole is particularly interesting in view of the fact that the former compound could not be shown to substitute for indole as a tryptophan precursor in *Claviceps*. Although glucose facilitated the uptake of anthranilic acid, no tryptophan was formed. Some essential condition in the form of a coenzyme, activator, or the like, necessary for anthranilic acid conversion, is probably lacking.

Failure of shikimic acid to serve as a tryptophan precursor might have been predicted from the behavior of anthranilic acid. Obviously, the former compound must occupy a position in the biosynthetic scheme prior to the latter substance. Since the pathway was apparently blocked between anthranilic acid and indole, assuming this portion of the biosynthetic scheme in *Claviceps* to be identical with that of *Neurospora*, no compound prior to anthranilic acid could possibly function as a tryptophan precursor. The role of shikimic acid remains to be determined in a strain of *Claviceps*, or other organism, capable of utilizing anthranilic acid for the synthesis of tryptophan.

Tryptophan disappeared from the *Claviceps* replacement cultures in a relatively short time. The optimum hydrogen ion concentration for tryptophan utilization or production was apparently so near the neutral point that substitution of water for the *p*H 7.5 phosphate buffer showed little quantitative effect. At the same time it was shown that phosphate did not materially influence the reactions. On the other hand, added serine definitely inhibited tryptophan utilization. It may be postulated that serine couples with one of the breakdown products of tryptophan (possibly indole) to produce additional tryptophan, thus maintaining a dynamic equilibrium in favor of that compound.

Although Yanofsky (12) reported that tryptophan was found to be an effective inhibitor of tryptophan desmolase of *Neurospora*, in this case the quantity of tryptophan added was probably insufficient to exhibit a definite inhibitory effect on the formation of additional tryptophan.

The doctrine of comparative biochemistry suggested that tryptophan would serve as a precursor to nicotinic acid in *Claviceps*, and evidence in support of this viewpoint was obtained.

The schematic outline above summarizes those biosynthetic reactions studied in *Claviceps*.

No lysergic acid derivatives were ever detected, either colorimetrically or chromatographically in the replacement cultures, even though formation of tryptophan could be stimulated by additions of indole and serine. It will probably be necessary to study the physiology and the biosynthetic abilities of *Claviceps* grown under normal parasitic conditions to determine the relationship between tryptophan and lysergic acid.

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Analgesic Effects of Clinically Useful Antiepileptics*

By EWART A. SWINYARD, DOUGLAS L. SMITH, and LOUIS S. GOODMAN

Eight clinically employed antiepileptic drugs were compared with morphine sulfate and acetylsalicylic acid for minimal neurological toxicity and for relative ability to prolong the reaction time of rats subjected to radiant-heat stimulation of the tail. The order of decreasing analgesic potency was: morphine, phenobarbital, phethenylate, mephobarbital, Mesantoin®, diphenylhydantoin, paramethadione, phenacemide, trimethadione, and acetylsalicylic acid. Except for morphine, acetylsalicylic acid, trimethadione, and phenacemide, all drugs exhibited neurotoxicity before analgesic activity.

MANY DRUGS exert multiple pharmacological effects on the central nervous system. For example, among the clinically useful anticonvulsants, phenobarbital is antiepileptic, sedative, hypnotic and, in large doses, anesthetic; diphenylhydantoin is antiepileptic and, in large doses, a central nervous system stimulant. Although trimethadione is now employed as an antiepileptic, it was originally explored clinically as an analgesic (1, 2). More recently, an analgesic effect has been attributed to phenacemide (3). Despite the indication that certain antiepileptic drugs may possess analgesic activity, no systematic evaluation of this activity has been reported. Accordingly, the analgesic properties and neurotoxicity of eight clinically employed antiepileptic drugs were determined in rats and compared with similar data for morphine sulfate and acetylsalicylic acid. The results obtained provide the basis for this report.

METHODS

Male albino Sprague-Dawley rats were employed as experimental animals. For the analgesic experiments, animals weighing 50–180 Gm. were used; in the toxicity studies, some animals weighed up to 300 Gm. All rats had free access to food and water except during the short time they were removed from their cages for testing. In order to avoid the development of tolerance, animals were not given repeated doses of the drug being tested and were allowed at least seven days to recover from the previously administered drug. The eight anticonvulsant drugs tested were as follows: phenobarbital sodium, mephobarbital (Mebaral®),[1] phethenylate sodium (Thiantoin® Sodium),[2] Mesantoin®,[3] diphenylhydantoin sodium (Dilantin® Sodium),[4] paramethadione® (Paradione®),[5] trimethadione (Tridione®)[6] and phenacemide (Phenurone®);[7] the two analgesic agents tested were morphine sulfate and acetylsalicylic acid. Mephobarbital, Mesantoin®, paramethadione, phenacemide, and acetylsalicylic acid were suspended in a 10% acacia solution and given orally; phenobarbital, phethenylate, diphenylhydantoin, trimethadione, and morphine sulfate were injected subcutaneously in aqueous solution.

Analgesic potency was determined by means of a radiant-heat apparatus similar to that originally described by Davies, Raventos, and Walpole (4), and modified by Chen (5, 6) to include a hollow rat-tail holder through which water was circulated to dissipate heat from the hot-wire heating unit. In addition, a variac and a voltmeter were placed in the circuit in order to control the line voltage to the instrument. When the animal's reaction time was being measured, the tail was manually held lightly but firmly over the heat source and the time required for tail withdrawal recorded. This procedure was employed in subjecting groups of six or more rats to various intensities of heat until several points were established on a regression line (ordinate, % tail withdrawal; abscissa, intensity of heat stimulus) which included at least one point representing failure of tail withdrawal in all animals in the group within *five seconds* and one point representing successful tail withdrawal in all animals in the group within the prescribed time. Logarithmic probability paper was used and the regression line visually fitted to the plotted points. From such plots of data the intensity of heat stimulus necessary to cause 50% of control rats to withdraw their tails within *five seconds* (HS_{50}) was established. This heat stimulus was found to be relatively independent of changes in the age and the weight of the rats, and it was used for all subsequent analgesic measurements. Analgesic activity was measured by determining the quantity of drug required to double the reaction time in 50% of rats exposed to the HS_{50}. Thus, the quantal end point employed for the measurement was failure of the animal to withdraw its tail during a *ten-second* exposure to the HS_{50}.

The criteria used for determining minimal neurological toxicity were the same as those previously described by us (7, 8). Briefly, minimal neurological deficit was determined by the following five tests: positional sense, righting, gait and stance, muscle tone, and equilibrium. Abnormal neurological status disclosed by any of these five tests was taken as the end point for the determination. However, if any other overt sign of toxicity consistently appeared after a dose lower than that causing neurological deficit, it was taken as the end point.

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[1] Supplied by Sterling-Winthrop Research Institute.
[2] Supplied by Dr. K. K. Chen, Eli Lilly & Company.
[3] Supplied by Harry Althouse, Sandoz Chemical Works, Inc.
[4] Supplied by Parke-Davis & Company.
[5] Supplied by Dr. R. K. Richards, Abbott Laboratories.

In the determination of analgesic activity and toxicity, groups of four to six animals were given various quantities of the drug under study and tested at the predetermined time of peak analgesic effect until at least three dose levels were established between no analgesia or toxicity and complete analgesia or toxicity. The results obtained were plotted on logarithmic probability paper and a regression line visually fitted to the plotted points. From such plots of the data, the dose of drug required to produce tail withdrawal in 50% of animals (ED_{50}) and the dose causing minimal neurological deficit in 50% of animals (TD_{50}) were determined and protective indices (TD_{50}/ED_{50} = P. I.) and 95% confidence limits calculated by the method of Litchfield and Wilcoxon (9).

RESULTS

The routes of administration employed for the various drugs and the times of their peak analgesic effect in rats are listed in Table I. It may be seen from the table that the time of peak analgesic activity varies from fifteen minutes for acetylsalicylic acid to 120 minutes for Mesantoin®. Except for acetylsalicylic acid and phenobarbital, the drugs administered orally have a longer peak time (60 minutes or more) than those given subcutaneously (30 minutes).

TABLE I.—ROUTE OF ADMINISTRATION AND TIME OF PEAK ANALGESIC EFFECT IN RATS

Drug	Route	Time of Peak Effect (Min.)
Phenobarbital sodium	s. c.[a]	60
Mephobarbital	per os[b]	60
Phethenylate sodium	s. c.	30
Mesantoin®	per os	120
Diphenylhydantoin	s. c.	30
Paramethadione	per os	60
Trimethadione	s. c.	30
Phenacemide	per os	60
Acetylsalicylic acid	per os	15
Morphine sulfate	s. c.	30

[a] s. c. = subcutaneous in aqueous solution
[b] per os = orally suspended in 10% acacia solution.

The comparative analgesic potencies of the compounds are shown in Fig. 1, from which it may be seen that all drugs tested have analgesic activity in rats as measured by the radiant-heat method. The ED_{50} varies from 1.9 mg./Kg. for morphine sulfate to 400 mg./Kg. for acetylsalicylic acid, and the ED_{50} of the eight anticonvulsant drugs are intermediate. Of the anticonvulsant drugs, phenobarbital is the most potent as an analgesic, whereas trimethadione and phenacemide are the least potent.

The neurotoxicities of the ten compounds, determined at the time of their peak analgesic effect, are shown in Fig. 2. The TD_{50} varies from 10.2 mg./Kg. for morphine sulfate to more than 1,600 mg./Kg. for acetylsalicylic acid. The anticonvulsant drugs are less toxic than morphine and more toxic than acetylsalicylic acid. Phenobarbital is the most toxic of the anticonvulsant drugs and trimethadione and phenacemide are the least toxic. For example, a dose of 22.0 mg./Kg. of phenobarbital produces evidence of minimal neurological deficit in 50% of rats, whereas a dose of 400 mg./Kg. of trimethadione or 380 mg./Kg. of phenacemide is required to produce the same level of toxicity. The order of toxicity of the other five anticonvulsant drugs is as follows: mephobarbital and phethenylate > Mesantoin® and diphenylhydantoin > paramethadione.

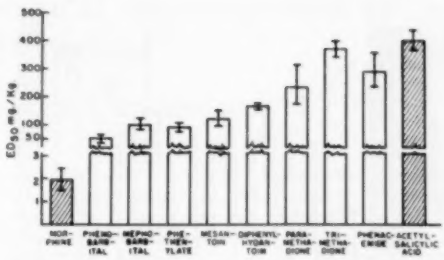

Fig. 1.—The analgesic potencies of some anticonvulsant and analgesic drugs.

The analgesic doses are expressed in mg./Kg. along the ordinate and the 10 drugs tested are listed along the abscissa. The ED_{50} for the 8 anticonvulsant drugs are represented by the open vertical bars and the ED_{50} for the 2 analgesic drugs by the lined bars. The 95% confidence limits are shown by the vertical bracketed lines.

The calculated protective indices (TD_{50}/ED_{50} = P. I.) for the ten compounds studied are shown in Fig. 3. Except for trimethadione and phenacemide, all anticonvulsant drugs have protective indices less than 1.0. The protective indices for trimethadione and phenacemide are 1.08 and 1.31, respectively, whereas those for morphine sulfate and acetylsalicylic acid are 5.37 and >4.0, respectively. Thus, it is apparent that only trimethadione and phenacemide exhibit analgesic activity in nontoxic doses and that their protective indices in rats are far below those of morphine and acetylsalicylic acid.

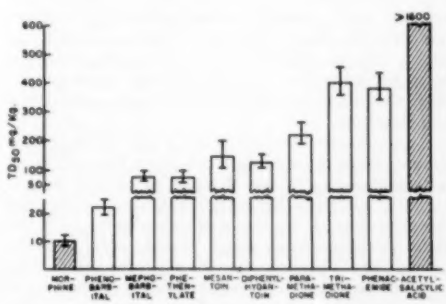

Fig. 2.—The toxicities of some anticonvulsant and analgesic drugs.

The toxic doses are expressed in mg./Kg. along the ordinate and the 10 drugs tested are listed along the abscissa. The open vertical bars indicate the TD_{50} for the 8 anticonvulsant drugs; the lined bars, the TD_{50} for the 2 analgesic drugs. The 95% confidence limits are shown by the vertical bracketed lines.

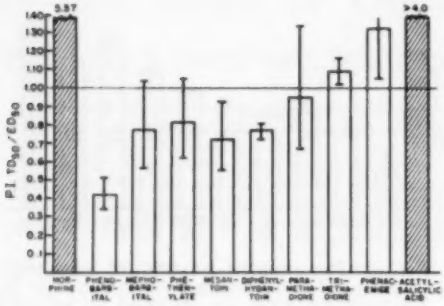

Fig. 3.—The protective indices (P. I.) of some anticonvulsant and analgesic drugs.

The protective indices are shown along the ordinate and the 10 drugs tested are listed along the abscissa. The open vertical bars indicate the P. I. for the 8 anticonvulsant drugs; the lined bars, the P. I. for the 2 analgesic drugs. The 95% confidence limits are shown by the vertical bracketed lines.

DISCUSSION

The data presented above show wide variation in the times of *peak analgesic effect*. The results are in agreement with observations previously reported by us (8) regarding variations in the time of *peak anticonvulsant effect* of these agents, and emphasize the importance of Loewe's discussion (10) of drug action and the necessity for making potency measurements at the time of peak effect rather than at arbitrarily fixed times after drug administration. Although the analgesic potencies reported herein were determined at the previously established time of peak analgesic effect for each drug, a critical comparison of analgesic potencies is valid only for those drugs given by the same route of administration.

All anticonvulsant drugs tested exhibited analgesic activity as measured by their ability to prolong the reaction time of rats subjected to radiant-heat stimulation of the tail. As analgesics, except for trimethadione, they are more potent than acetylsalicylic acid; indeed, phenobarbital is approximately eight times more potent than acetylsalicylic acid. Clearly, morphine is in a class by itself; it is approximately twenty-seven times more potent than phenobarbital and 195 times more potent than trimethadione.

Although the absolute toxicity and potency of a drug contribute valuable information for predicting its usefulness, a factor of equal or greater importance is the ratio between the toxic dose and the effective dose, i. e., the protective index. Of the eight anticonvulsant drugs tested, only trimethadione and phenacemide have protective indices greater than one. This is particularly interesting in view of the fact that trimethadione was originally tested clinically as an analgesic (2) and an unusual case of loss of pain sense (3) has been attributed to phenacemide. Also, trimethadione has been reported selectively to depress polysynaptic transmission in the spinal cord (acute spinal cats) (11). Whether the other anticonvulsants have a similar action has not been determined.

It is interesting to compare the analgesic activity of these drugs with their previously reported anticonvulsant potency. The compounds most potent as anticonvulsants, as measured by a battery of tests in mice and rats (8), also have the highest analgesic activity as measured by the radiant-heat method employed herein. Thus, phenobarbital is the most potent of the antiepileptic series whether tested as an analgesic or as an anticonvulsant, whereas paramethadione, trimethadione, and phenacemide are least potent as measured by the same tests. Nevertheless, this correlation is not sufficiently good to warrant the use of analgesic data for predicting experimental anticonvulsant specificity.

SUMMARY

Eight clinically employed antiepileptic drugs were compared with morphine sulfate and acetylsalicylic acid for minimal neurological toxicity and for relative ability to prolong the reaction time of rats subjected to radiant-heat stimulation of the tail. The heat stimulus necessary to cause tail withdrawl in 50% of control rats within five *seconds* (HS_{50}) was determined; this intensity of heat was employed for all subsequent analgesic tests. Analgesic activity (ED_{50}) was measured by determining the quantity of drug required to double the reaction time in 50% of rats exposed to the HS_{50}.

The ED_{50} and 95% confidence limits for morphine sulfate and acetylsalicylic acid were 1.9 (1.5–2.4) mg./Kg. and 400 (367–436) mg./Kg., respectively. The ED_{50} for the eight anticonvulsant drugs were intermediate to these values. The order of decreasing analgesic potency was: morphine > phenobarbital > phethenylate > mephobarbital > Mesantoin® > diphenylhydantoin > paramethadione > phenacemide > trimethadione > acetylsalicylic acid. Except for morphine, acetylsalicylic acid, trimethadione, and phenacemide, all drugs exhibited neurotoxicity before analgesic activity.

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A Preliminary Phytochemical Investigation of *Digitalis ferruginea**

By ROBERT M. APPEL† and OLE GISVOLD‡

The cardiac glycosides of the fresh first-year leaves of *Digitalis ferruginea* have been resolved into alpha acetyl digitoxin and an amorphous glycoside whose properties resemble digitoxin. The saponin tigonin was also very readily isolated in the amount of about two per cent of the dry weight of the leaves.

THE ISOLATION of the acetyl-digoxins from the first-year fresh leaves of *Digitalis lanata* provided the stimulus for the phytochemical investigation of the first-year fresh leaves of other species of Digitalis grown in Minnesota. These studies have been aided, in part, by newer, simplified enzyme-inhibiting techniques that have been previously published (1). A further stimulus for these studies has been the report that the oral absorption, in cats, of cardiac glycosides is closely correlated with their partition coefficients (2). Therefore, search for other acetyl-cardiac glycosides (predicted high partition coefficients) was desirable. It was hoped that the acetyl-digoxins could be found in some of the Digitalis species. These glycosides, prior to beginning these studies, had not been isolated *per se* in nature. They had only been prepared by the enzymatic degradation of lanatoside A which is obtained from the second-year leaves of *Digitalis lanata*. After our studies had been initiated, Stoll and Renz (3) reported the presence of β-acetyl digitoxin, lanatoside A, and lanatoside B in the second-year dry leaves of *Digitalis ferruginea* that were collected in Asia Minor. They had failed to find these glycosides when this plant was grown in Switzerland.

EXPERIMENTAL

Primary Extract.—A primary aqueous extract of 9,436 Gm. of fresh leaves (about 80% moisture) of *Digitalis ferruginea*, grown in Minnesota in the summer of 1952, was prepared according to previously published procedures. Filtration of the coagulated leaves was markedly simplified by using a fine wire, 200-mesh, Monel metal screen. No filter cell was necessary. An occasional cleaning of this screen was all that was required for smooth operation. The marc was temporarily preserved and was also examined.

Ketone Extract.—Extraction of the aqueous extract, with methyl isobutyl ketone (MIBK) yielded some clear MIBK extract plus some emulsified MIBK and an "aqueous mother liquor." The emulsion was best cracked by centrifuging at 1,500 r. p. m. for twenty minutes. During the MIBK extraction, an insoluble, solid, brown interphase was obtained which was collected, dried, and labeled "interphase material." Concentration of the MIBK extract under a vacuum at 50–55° yielded a green tarry-like residue that was digested with petroleum ether several times to defat it. The petroleum ether-insoluble residue was digested with ether to give chiefly "ether-soluble material" and a smaller amount of a tan solid labeled "ether-insoluble material."

Ether-Insoluble Material.—This material was dissolved in a minimum amount of methyl isobutyl ketone warmed with a small amount of charcoal together with filter cell (celite) and the mixture filtered. After removal of the solvent, the resultant light yellow solid was washed with very small amounts of cold methyl ethyl ketone (constant boiling mixture with water, MEK-HOH) to give a MEK-HOH white insoluble solid, 300 mg., and a MEK-HOH soluble fraction 1.75 Gm.

MEK-HOH Insoluble Material.—This fraction, after recrystallization from 50% aqueous methanol, gave colorless needles that melted at 215–217° when dried at 140° in a vacuum. A mixed melting point with either α- or β-acetyl digitoxin[1] showed no depression. The Keller-Kiliani test showed a gold-brown ring at the interphase with a blue color in the acetic acid layer. The presence of digitoxose was verified by the following test. A standard loopful of an alcoholic solution of this crystalline glycoside was placed on Whatman No. 1 paper and allowed to dry. The paper was then sprayed with a solution of the following composition: 2.8 cc. hydrochloric acid, 10 drops of a 10% solution of ferric chloride, and enough methanol to bring the volume to 100 cc. (Gregg and Gisvold Reagent[2]). The paper was subsequently placed in front of four 250-watt infrared lamps at a distance of 35 cm. and heated for ten minutes. A typical blue-gray spot that is positive for digitoxose developed (Gregg and Gisvold test[2]). A negative test, i. e., no colorless spot, for a 1,2-glycol was obtained by the following test (periodate-benzidine test[3]). A standard loopful *loc. cit.* of an alcoholic solution of the crystalline glycoside was placed on Whatman No. 1 paper and allowed to dry. The paper was then sprayed with a saturated

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[1] These glycosides were obtained through the courtesy of Dr. A. Stoll, Basel, Switzerland.
[2] See Gregg and Gisvold, THIS JOURNAL, 43, 106(1954).
[3] Personal communication with F. Smith, University of Minnesota.

solution of potassium periodate. After one minute, the paper was sprayed with a solution of the following composition: 10 parts of ethanol and 20 parts of acetone and 1 part of 0.2 *N* hydrochloric acid.

The rotation of a 0.0118-Gm. sample of this crystalline glycoside (dried at 100° in a vacuum) in 0.7 cc. methanol observed in a 0.5-diam. micropolarimeter tube was 0.207°; $[\alpha]_D^{25} = +24.6$. The rotation of a 0.0148-Gm. sample in 0.7 cc. pyridine observed in a 0.5-dm. micropolarimeter tube was 0.05; $[\alpha]_D^{25} = +4.7$. The reported values for α-acetyl digitoxin are +24.5 and +4.8 respectively (3).

Anal.—Calcd. for acetyl digitoxin $C_{43}H_{66}O_{14}\cdot H_2O$: C, 62.60; H, 8.31; $COCH_3$, 5.22. Found: C, 62.45; H, 8.16; $COCH_3$, 5.69.

Deacetylation of the crystalline glycoside by the method of Stoll and Renz (3) gave an acetyl free product that, after several recrystallizations from 50% aqueous methanol, gave no depression when a mixed melting point with digitoxin was taken.

MEK-HOH Soluble Material.—A Keller-Kiliani test on this material gave a red-brown ring at the interphase and a blue color in the acetic acid. A portion (665 mg.) of this material was partitioned between 16% aqueous methanol and chloroform. From the chloroform layer, 630 mg. of glycosidic material was recovered. Most of the colored material was found in the aqueous methanol layer. The chloroform-soluble glycosidic material was crystallized from a mixture of equal parts of methanol and chloroform to which a few drops of anhydrous ether were added. Colorless plates were obtained that melted at 214–216°. A mixed melting point with the above-described crystalline glycoside or with either α- or β-acetyl digitoxin showed no depression. X-ray analysis[4] indicated that the two crystalline glycosides were identical.

Ether-Soluble Material.—This material had a dark green tarry-like appearance and gave a positive Keller test, the interphase being a red-brown color. Extraction of this material with petroleum ether and with benzene left a green solid. This green solid was dissolved in a large volume of anhydrous ether. The addition of petroleum ether effected the separation of a light green precipitate which was dissolved in ethanol and passed through a column of aluminum oxide (Chromatographic Adsorption Grade Alumina, Merck). The green pigments were adsorbed at the top of the column. Washing the column with ethanol did not remove the dark pigments. The eluates upon evaporation left a light yellow solid that gave a strong Keller test. This solid was extracted with anhydrous ether in a Soxhlet. A small volume of hexane was added to the ether extract until the mixture was turbid. Upon cooling, some white solid material separated. The filtrate was concentrated until it became turbid and again cooled with the separation of more white solids. This procedure was repeated until no further separation of white solids occurred. The mother liquor was yellow in color and upon removal of the solvent yielded a brownish viscous solid that gave a negative Keller test.

The combined white amorphous solids weighed 4.78 Gm. and gave a strong positive Keller-Kiliani test free of red color at the interphase. Attempts to obtain this glycosidic material in a crystalline state failed. Partitioning between 16% aqueous methanol and chloroform did not produce a crystallizable fraction. The glycosidic material was dissolved in 500 cc. of reagent chloroform and then passed through a column of aluminum oxide (150 Gm. in a glass tube with an inside diameter of 2.4 cm.). Elution with chloroform yielded 90 mg. of resinous material that gave a negative Keller-Kiliani test. Elution with 95.5 parts of chloroform and 4.5 parts of methanol yielded 530 mg. of a light yellow solid that gave a positive Keller-Kiliani test with no red at the interphase. All attempts at crystallization produced amorphous glycosides. Elution with methanol yielded about 4 Gm. of noncrystallizable glycosides that gave a positive Keller-Kiliani test containing no red at the interphase. The most highly purified product melted at 140–145°. The presence of digitoxose was confirmed by the Gregg and Gisvold test. The periodate benzidine test for a 1,2-glycol was positive, and no acetyl value was obtained. The R_f value of this amorphous glycoside was the same as the crystalline glycosides, i. e., 0.96 when the solvent was a constant boiling mixture of methyl ethyl ketone and water and 0.90 when the solvent mixture was methyl isobutyl ketone, methanol, and water in the ratio of 10:1.8:50.

Anal.—Calcd. for digitoxin $C_{41}H_{68}O_{13}\cdot 2H_2O$: C, 61.47; H, 8.55. Found: C, 61.38; H, 8.35.

Examination of the Marc.—A 50% aqueous ethanol extract of the marc upon careful examination yielded a total of about 660 mg. of purified glycosides whose properties were quite identical to those of the amorphous glycoside.

Interphase Material.—Since Stoll and Renz (3) found the majority of their glycosides (lanatoside A) in the insoluble interphase between water and chloroform during partitioning after lead salt purification, the "interphase material" obtained in this investigation by partitioning between water and methyl isobutyl ketone was investigated. No glycosides were found.

Examination of Aqueous Mother Liquor.—This fraction (27 gallons) was extracted with *n*-butanol by means of 4-L. separatory funnels. Any emulsions that were obtained were broken with the aid of sodium sulfate and centrifuging at 1,500 r. p. m. Approximately 5.5 gallons of a clear yellow-green *n*-butanol extract were obtained. The *n*-butanol extract was concentrated under reduced pressure at 50–60° to a volume of 200 cc. from which 23.93 Gm. of a light-colored amorphous solid was obtained. The filtrate was labeled "*n*-butanol mother liquor."

The amorphous solid was soluble in water with foaming and opalescence. It was soluble in ethanol and hot *n*-butanol but was insoluble in the following solvents: cold *n*-butanol, hot chloroform, hot benzene, hot ether, and hot petroleum ether. Some of the qualitative tests for characterization may be summarized as follows: (*a*) Keller reaction, negative; (*b*) Gregg and Gisvold test for digitoxose, negative; (*c*) periodate-benzidine test for 1,2-glycols, positive; (*d*) Molish test, positive; (*e*) Seliwanoff test, negative; (*f*) negative, test with Barfoed reagent; (*g*) negative, ferric chloride test; (*h*) negative, Lieberman-Burchard test for sterols; (*i*) the addition of ether to a 5% aqueous solution of the amorphous solid yielded a gelatinous precipi-

[4] Through the courtesy of Dr. W. Lipscomb, Physical Chemistry Department, University of Minnesota.

tate after one hour (saponins); (*j*) weak positive cholesterol addition complex saponide (tigonin); (*k*) precipitated from 50% aqueous ethanol by an aqueous solution of basic lead acetate; (*l*) the addition of a few drops of concentrated sulfuric acid to a saturated aqueous solution of the amorphous solid produced a deep red by transmitted light and a violet color by reflected light. The preceding tests indicated that this amorphous fraction was a saponin. Attempted crystallization from *n*-butanol 50% ethanol or ethanol only gave an amorphous product that softened at 215° and melted between 242–250° with decomposition.

Hydrolysis of the above-described saponin with hydrochloric acid (4) gave a crystalline sapogenin; however, paper chromatographic analysis of the sugars gave a distorted and erroneous picture. The saponin was therefore hydrolyzed with much better results as follows: 250 mg. was dissolved in 5 cc. of ethanol and 10 cc. of water, to which 0.86 cc. of concentrated sulfuric acid was added. The mixture was refluxed for two and one-half hours. The foaming that occurred was controlled by the addition of 2 cc. of *n*-amyl alcohol. The reaction mixture contained a white solid and was yellow in color as contrasted with the red-brown colors and tar-like appearance when hydrochloric acid was used. The insoluble white solid was collected and recrystallized twice from methanol to give colorless needles. It melted at 205–208° and did not depress the melting point of the genin that was obtained by hydrolysis with hydrochloric acid. When crystallized from acetone, the melting point was 203–204°. Reported (5) melting point for tigogenin is 203–204° from acetone. The rotation of a 0.0136-Gm. sample of this genin in 0.7 cc. of pyridine observed in a 0.5-dm. micropolarimeter tube was −0.48°; $[\alpha]_D = -49.4$. Reported (5) value for tigogenin is −49. Acetylation with acetyl chloride in dry pyridine gave an acetate (crystallized from 90% ethanol) that melted at 198–200°. Reported (5) value for tigogenin acetate is 200–202°. Benzoylation with benzoyl chloride in dry pyridine gave a benzoate (recrystallized from acetone) that melted at 222–224°. Reported (5) for tigogenin benzoate 224–225°. A digitonide was obtained that melted at 225–242°.

Anal.—Calcd. for tigogenin $C_{27}H_{44}O_3 \cdot 1.5H_2O$ (dried at 100° for two hours at 5–6 mm.): C, 73.09; H, 10.70. Found: C, 73.42; H, 10.55. Calcd. for tigogenin $C_{27}H_{44}O_3 \cdot 1H_2O$ (dried at 140° for five hours at 20 mm.): C, 74.61; H, 10.67. Found: C, 73.95; H, 10.48.

Identification of the Sugar Residues of the Saponin (Tigonin).—After removal of the sapogenin from the hydrolysis mixture, described above, the mixture was neutralized with barium carbonate and filtered. The filtrate was concentrated to a syrupy mass which was dissolved in ethanol and filtered to give a light yellow solution. A standard loopful (*loc. cit.*) of each of the following solutions was placed at the top of two strips of Whatman No. 1 paper and allowed to dry: sugar solution from the hydrochloric acid hydrolysis, the sugar solution from the sulfuric acid hydrolysis, and a mixture of these two sugar solutions. The developing solution used was *n*-butanol, distilled water, and glacial acetic acid in a ratio of 4:5:1. The papers (in the same jar) were saturated with the stationary phase forty-five minutes. The paper was developed with the mobile phase for ten hours at 25–26°. The solvent front had moved 30 cm. After drying, the paper was sprayed with the periodate-benzidine reagents. The following R_f values (Table I) were calculated for the colorless spots that appeared on the blue-gray background:

TABLE I.—R_f VALUES

	R_f Values				
	1	2	3	4	5
Sugars from HCl hydrolysis					
Paper 1	...	0.19	0.28	0.47	0.67
Paper 2	...	0.21	0.29	0.49	0.71
Sugars from H_2SO_4 hydrolysis					
Paper 1	0.12	0.20	0.29	...	...
Paper 2	0.12	0.22	0.31	...	...
Average R_f values	0.12	0.21	0.29	0.48	0.69
Mixture of sugars from mild and strong hydrolysis	0.11	0.21	0.30	0.49	0.71

The experimental data show the presence of two products from the hydrochloric acid hydrolysis that have abnormally high R_f values for sugars. This may be due to secondary changes that were caused by the hydrochloric acid. Furthermore, a new sugar having a lower R_f value was obtained by the sulfuric acid hydrolysis.

From a number of paper chromatographic analyses, using known sugars and the unknown sugars obtained by the hydrolysis of the saponin performed as described above, Table II has been compiled to summarize the experimental findings.

TABLE II.—SUMMARY OF EXPERIMENTAL FINDINGS

	Average R_f Values		
Unknown sugar mixture	0.12	0.21	0.30
Maltose	0.12	...	...
D-Glucose	...	0.23	...
D-Galactose	...	0.20	...
D-Xylose	...	...	0.30
Mixture of all above	0.12	0.21	0.29

It may be concluded from the experimental findings that a mixture of glucose and galactose in decreased concentrations did not separate, and the R_f value of this mixture closely paralleled the R_f value of 0.21 obtained on the same paper for the unknown sugar mixture. It can also be concluded from experiments performed upon successively decreasing concentrations of the unknown sugars that the largest percentages of sugars in the unknown sugar mixture were principally those giving the intermediate R_f value. Although maltose gave an R_f value identical to the first unknown sugar from which it would not separate, it is well known that the R_f values of a number of disaccharides are very similar. Furthermore, the conditions of hydrolysis should eliminate the presence of maltose. It therefore must be classified for the present as an unidentified disaccharide (perhaps cellobiose) that perhaps may contain a β-configuration at C_1 of one of the sugar

TABLE III.—SUMMARY OF PAPER CHROMATOGRAPHIC TESTS

	R_f Values			
	1	2	3	Average
Lanatoside C	...	...	0.189	0.19
A crude glycoside mixture from *Digitalis lanata*	0.347	...	...	0.35
	0.511	...	0.502	0.51
Digoxin	0.843	...	...	0.84
Digitoxin	0.848	0.902	0.894	0.88
Acetyl digoxin	0.892	0.884	0.900	0.89
Digitalis ferruginea glycosides				
Crystalline glycosides	0.910	0.908	0.901	0.91
Amorphous glycosides	0.881	...	0.901	0.89

residues. The latter conclusion is based upon the ease of hydrolysis of α-glycosidal linkages compared with the β-glycosidal linkages.

The presence of galactose was confirmed by oxidation of the sugar mixture with nitric acid (sp. gr. 1.15) to give mucic acid whose melting point showed no depression when it was mixed with an authentic sample of mucic acid.

Xylose was definitely shown to be present.

Paper Chromatographic Studies.—Paper chromatography was utilized as a tool in an attempt to assist in the characterization of the glycosides of *Digitalis ferruginea*. The solvent mixtures previously reported in the literature for use in conjunction with cardiac glycosides proved ineffective. The solvent mixture of Svendsen and Jensen (6) composed of chloroform, methanol, and water in the ratio of 10:4:5 gave erratic flow or even frequently appeared to reverse its direction even when tried at 20°, 22°, 24°, 26°, 28°, and 30°, respectively. The glycosides tested moved only a few centimeters from the point of application with no separation of the component mixtures. The substitution of methyl isobutyl ketone (MIBK) for chloroform with an adjustment of the methanol concentration resulted in the development of the following mixture: 10 MIBK, 1.8 methanol, and 5 water. The mobile phase was allowed to act for four to five hours at 20–21° after which time it had moved 37 cm. The position of the glycosides was detected by the Gregg and Gisvold test, *loc. cit.* Table III summarizes the results obtained.

When a mixture of digitoxin, acetyl digoxin, and the glycosides of *Digitalis ferruginea* was chromatographed with the same solvent, i. e., MIBK, methanol, and water, 10:1.8:5, no separation could be effected.

Substitution of a constant boiling mixture of methyl ethyl ketone and water as the mobile phase from the above described mobile phase gave the results found in Table IV.

As in the preceding case, the same mixture of glycosides gave one R_f value of 97 and therefore could not be resolved qualitatively on paper.

TABLE IV.—R_f VALUES OF GLYCOSIDES

	R_f Values
A crude mixture of *Digitalis lanata* glycosides	0.75
Digoxin	0.92
Digitoxin	0.95
Acetyl digoxin	0.95
Crystalline glycosides from *Digitalis ferruginea*	0.95

Attempted Resolution of the Amorphous Glycosides.—A portion of the amorphous glycosides, 200 mg., was dissolved in 10 cc. of constant boiling methyl ethyl ketone and water and placed at the top of a cellulose powder[5] (165 Gm.) column. The column was developed and eluted with the above solvent until 100 test tubes (in an automatic fraction collector) containing 2.5 cc. each of the solvent were obtained. The glycosides were found in tubes 11 through 16 with maximum concentration in 13 and 14. When the crystalline glycosides from the *Digitalis ferruginea* were substituted for the amorphous glycosides, much the same results were obtained.

Sensitivity of the Gregg and Gisvold Test.—A 0.25% solution of acetyl digoxin in 50% aqueous methanol was used to make the following concentrations: 0.125, 0.06, 0.03, 0.015, and 0.008%, respectively. A negative Gregg and Gisvold test was obtained with the 0.008% concentration, whereas all other concentrations gave a distinct blue-gray spot. When the ferric chloride was not included with the 1% hydrochloric acid, exactly the same results were obtained. The presence of ferric chloride, however, provided a slight color so that it was easier to see where the paper had been sprayed and also gave better contrast in color to aid in the location of the spot.

Partial Range of the Gregg and Gisvold Test.—Roughly, 1% solutions in methanol of digoxin, acetyl digoxin, digitoxin, acetyl digitoxin, lanatoside A, lanatoside C, gitoxin, crystalline and amorphous glycosides of *Digitalis ferruginea*, gitalin, digoxigenin, and digitoxose were prepared. A standard loopful (platinum wire measuring 2 mm. outside diameter and 1.5 mm. inside diameter) of each of these compounds was subjected to the Gregg and Gisvold test. A positive test, blue-gray spot, was obtained for each compound with the exception of digoxigenin and the blank (the solvent and methanol). This test appears to be generally applicable for the detection of the Digitalis cardiac glycosides. The presence of the genin can be detected by the characteristic fluorescence in a darkroom with an ultraviolet lamp upon the same test spots.

Use of Periodate-Benzidine Reagent on Some Digitalis Cardiac Glycosides.—Roughly, 1% solutions in methanol of the compounds listed in the previous paragraph were subjected to the periodate-benzidine test. A standard loopful was used in each case. A positive test, a colorless spot, was obtained only with digoxin, ditoxin, and amorphous glycosides of *Digitalis ferruginea*. Ringing (i. e.,

[5] Whatman ashless powder cellulose for chromatography standard grade.

the formation of a colorless band around a blue-gray spot), which frequently occurred when 2 or 3 loopfuls of solution were added over each other, should not be mistaken for a positive test. The blanks also showed ringing with 2 and 3 loopfuls. Those compounds that gave a negative clear spot after one minute following the application of the reagents, did give a light blue spot upon standing overnight at room temperature. This phenomenon did not occur with those compounds that gave a positive test after one minute. The blue spot did not appear on the blank which retained the color of the background.

SUMMARY

1. A total of 7.53 Gm. (0.8%) of glycosides has been isolated from 9,436 Gm. of fresh leaves of *Digitalis ferruginea*. Of this total weight of glycosides, 2.05 Gm. was crystalline and 5.48 Gm. was amorphous.

2. The crystalline glycoside was identified as *alpha* acetyl digitoxin.

3. The amorphous glycosides exhibited properties that point to a digitoxin type compound.

4. A total of 39.13 Gm. (0.42%) of saponins was isolated which, upon hydrolysis, yielded tigogenin, glucose, galactose, and xylose and a disaccharide which might indicate that incomplete hydrolysis had occurred. The saponins are probably chiefly tigonin.

5. The Gregg and Gisvold test was shown to be sensitive to 0.015% concentrations of the Digitalis cardiac glycosides tested.

6. A partial range of the Gregg and Gisvold test is reported.

7. The qualitative application of the periodate-benzidene reagent to some Digitalis glycosides is reported.

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The Newer Anticholinergic Agents

II. Effectiveness as Antisialogogues*

By ARTHUR G. ZUPKO and LEON D. PROKOP

Quantitative collection of human parotid saliva by capping Stenson's duct and of canine saliva by Wharton duct cannulation before and after drug administration is shown to be a good objective indicator of the side effects of the newer anticholinergic agents. Antrenyl®, Pamine®, Pro-Banthine®, Banthine®, Prantal®, and Darstine® are compared by these means. The correlation between the antisialogogue action and side effects of these agents is discussed.

RECENTLY Armer (1) reported on the use of Banthine® as a premedication with sodium thiopental as the anesthetic in 599 cases of dental or oral surgery. In the author's opinion, Banthine® satisfactorily prevented laryngospasm and also, because of its effectiveness in suppressing gastric motility and free acid secretion, prevented nausea. Banthine® also exerted a profound antisialogogue effect so that less contamination of wounds occurred.

More recently Kirsner and Palmer (2), in a study of the newer gastric antisecretory compounds, claimed that the estimation of saliva output before and at intervals after the administration of an anticholinergic drug was the most objective indicator of side effects.

Recognition of the importance of antisialogogue action in the use of anticholinergic drugs prompted us to investigate the comparative effectiveness of Antrenyl®, Pamine®, Pro-Banthine®, Banthine®, Darstine®, and Prantal® in suppressing salivation. It seemed desirable to correlate further the relationship between the antisialogogue action and side effects exhibited by the anticholinergic drugs.

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EXPERIMENTAL

Methods.—Quantitative estimations of salivary output were performed on both human beings and dogs.

A.—Human Beings.—The parotid salivary output of 174 volunteer males was determined before and at half-hour intervals after administration of Antrenyl®, Pamine®, Pro-Banthine®, Banthine®, Prantal®, and Darstine®. Many of the same subjects were tested with more than one of the anticholinergic agents. In general, the procedure followed was to induce salivation in the subject by the chewing of one-half of a stick of gum for five minutes. The normal flow of parotid saliva was collected and measured quantitatively during this period by a method previously described by Curby (3) in which a methyl methacrylate polymer collection chamber with annular rings is centered over Stenson's duct, suction applied, and the flow of saliva collected by means of a polyethylene tubing leading from the collection chamber. The subject was then given twice the recommended single oral dose of the drug under test and the process of inducing salivation and collecting the flow during a five-minute period was repeated at half-hour intervals. Twenty-nine subjects were used to study the effect of placebo administration on salivary flow in the same manner as described.

B.—Dogs.—The comparative antisialogogue action of the anticholinergic drugs was determined in 31 dogs. The animals were anesthetized with 50 mg./Kg. of sodium pentobarbital given intramuscularly. Wharton's duct was cannulated with a fine glass cannula to which polyethylene tubing was attached. This tubing was then directed over the interval between two platinum electrodes of a universal drop recorder, the latter designed for use with all ordinary liquids and described elsewhere (4). Salivation was induced by the subcutaneous injection of 300 μ/Kg. of pilocarpine HCl and the flow of saliva recorded by means of a drop counter connected to a kymograph. The salivary output was measured quantitatively for ten minutes after stimulating saliva flow. An appropriate dose of the anticholinergic drug under test was administered intramuscularly and the effect on salivation observed. The complete cessation of salivary flow for a period of more than one hour was taken as an indication of maximum antisialogogue effectiveness.

RESULTS

A.—Human Beings.—The results of administration of the anticholinergic drugs on human parotid salivary outflow are shown in Table I. From this table it will be noted that, under the experimental conditions stated, Banthine® possesses the most potent antisialogogue action followed by Antrenyl®, Pro-Banthine®, Darstine®, Pamine®, and Prantal® in that order. Complete cessation of flow was observed most often with Banthine® and Antrenyl® but was not seen with either Darstine® or Prantal® in any of the subjects under study at the dosage levels indicated. With the exception of Pamine®, none of the drugs exhibited further significant suppression of saliva beyond the ninety-minute test period. It is probable that Pamine® has a far more potent antisialogogue action than could be indicated within the scope of the present study.

The deviation from normal salivary flow did not vary more than 4% in control subjects who were administered placebo tablets.

B.—Dogs.—The effect of varying doses of the anticholinergic agents on suppression of saliva in dogs pretreated with pilocarpine is shown in Table II. It may be seen that the antisialogogue activity of these drugs, under the experimental conditions described, varies greatly. Banthine®, for example, possesses an antisialogogue action 120 times greater than Darstine®, although both drugs have the same therapeutic dose. Prantal®, which has twice the therapeutic dose of Banthine®, is 70 times weaker in its antisialogogue action. Banthine® is used therapeutically in doses 10 times as great as Antrenyl® and 20 times as great as Pamine® but has approximately twice the potency of these drugs in suppressing salivation. The therapeutic dose of Banthine® is more than 3 times greater than that of Pro-Banthine®, but the antisialogogue activity is 4 times greater. Use of a larger number of dogs would conceivably alter the antisialogogue potency ratio of these drugs.

TABLE I.—EFFECT OF ANTICHOLINERGIC DRUGS ON HUMAN PAROTID SALIVARY FLOW

Drug and Dosage	No. Tested	Predrug Saliva Collected	Postdrug Saliva Collected			Maximum % Salivary Suppression
			30 Minutes	60 Minutes	90 Minutes	
Antrenyl®, 10 mg.	47	1.21[a,b] (0.31–4.15)	1.04 (0.00–3.80)	0.28 (0.00–2.71)	0.41 (0.00–3.13)	76.9
Pamine®, 5 mg.	51	1.32 (0.28–3.92)	1.34 (0.31–4.28)	1.23 (0.26–3.92)	1.00 (0.04–2.69)	24.2
Pro-Banthine®, 30 mg.	43	1.08 (0.41–4.22)	0.84 (0.11–3.96)	0.30 (0.00–3.14)	0.57 (0.00–3.02)	63.1
Banthine®, 100 mg.	36	1.34 (0.32–3.78)	0.11 (0.00–1.34)	0.16 (0.00–1.09)	0.13 (0.00–0.93)	91.8
Prantal®, 200 mg.	41	1.41 (0.48–5.13)	1.29 (0.32–4.81)	1.23 (0.21–4.34)	1.31 (0.30–4.41)	12.9
Darstine®, 100 mg.	44	1.07 (0.26–4.83)	0.66 (0.21–3.43)	0.99 (0.30–3.97)	1.01 (0.29–4.51)	38.7
Controls (Placebo)	29	1.19 (0.38–3.94)	1.22 (0.29–4.11)	1.18 (0.23–4.01)	1.24 (0.31–3.71)	...

[a] Each sample collected over a five-minute period.
[b] Values not in parentheses represent average volume in ml. Figures in parentheses represent the range in volume obtained.

TABLE II.—EFFECT OF VARYING DOSES OF ANTICHOLINERGIC DRUGS ON SALIVARY SUPPRESSION IN DOGS PRETREATED WITH PILOCARPINE

Drug and Dosage in μ/Kg.	No. Dogs	Average Percentage Inhibition of Saliva — Minutes after Injection			
		30	60	90	120
Banthine®					
5	2	73.3	64.5	66.9	50.3
10	2	100.0	100.0	100.0	100.0
Darstine®					
800	2	77.8	56.8	31.4	18.9
1200	3	100.0	100.0	91.3	86.4
Antrenyl®					
10	3	92.4	76.1	40.7	28.9
15	3	100.0	100.0	100.0	96.3
Prantal®					
500	2	67.2	53.9	37.6	28.9
700	2	100.0	100.0	82.1	57.4
Pro-Banthine®					
10	1	31.4	23.9	14.8	11.6
20	1	46.6	52.3	34.9	18.7
30	2	71.8	38.7	51.0	29.1
40	2	100.0	100.0	96.2	87.4
Pamine®					
10	3	42.1	38.7	50.4	58.6
20	3	100.0	100.0	100.0	100.0

DISCUSSION

It is interesting to note that results in both human beings and dogs indicate Banthine® and Antrenyl® to be the most effective antisialogogues. These two drugs also have the greatest incidence of side effects as noted in a previous study (5). Prantal® and Darstine® have a comparatively weak antisialogogue action and exert a minimum of side effects. Pro-Banthine® and Pamine® both have intermediate antisialogogue activity and incidence of side effects. It appears that the anticholinergic agents with the greatest effectiveness in suppressing salivation also exhibit maximum side effects. The antisialogogue effect of the anticholinergic drugs may, therefore, be an objective indicator of side effects.

Our experience with collecting human parotid saliva by capping Stenson's duct was not entirely satisfactory. Attempts to cap both the right and left duct simultaneously met with little success. Subjects complained of difficulty in chewing and collection of saliva was somewhat irregular. Consequently our results are based on the salivary outflow of only one duct. The possibility that the capping of one duct may significantly influence the flow of saliva from the contralateral duct exists.

The size of Stenson's duct in the subject was not related to the volume of parotid salivary flow of the duct. In some cases difficulty was encountered in finding the duct. Wiping the inner cheek area opposite the second upper molar dry with gauze and awaiting the appearance of a drop of saliva obviated this difficulty.

Gum chewing to induce salivation was preferred by subjects in lieu of warmed paraffin. We could find no significant difference in volume of saliva flow induced by these two agents. For the sake of uniformity, all subject chewed the same amount and brand of gum.

SUMMARY

1. Quantitative estimations of the parotid salivary output of 174 volunteer males were performed before and at half-hour intervals after administration of anticholinergic agents. Under the experimental conditions described, Banthine® possessed the most potent antisialogogue action, followed by Antrenyl®, Pro-Banthine®, Darstine®, Pamine®, and Prantal® in that order.

2. The comparative antisialogogue action of the anticholinergic drugs was determined in 31 dogs with results somewhat comparable to those obtained with human beings.

3. The antisialogogue activity of an anticholinergic drug has been shown to be a good objective indicator of side effects.

4. It is apparent that there will be a wide variation in the antisialogogue response of different individuals to the same dose of an anticholinergic drug. It would be logical to assume, therefore, that this same variation in the occurrence of side effects will exist.

5. Much progress has been made in the search for effective anticholinergic agents with minimal side effects since the introduction of Banthine®, although no existing drug of this type is completely devoid of side effects.

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Effects of Chronic Heat and Humidity Excess Upon Dermal Permeability*

By MICHAEL M. CLAY† and JOHN W. NELSON‡

The effect of prolonged treatment with high environmental temperature and humidity upon dermal permeability of the rat was studied. The decrease in permeability which resulted was not altered by adrenalectomy and was altered to a relatively minor degree by elimination of the unfavorable environmental factors.

NUMEROUS STUDIES have indicated that the secretion from the adrenal glands produces a marked decrease in connective tissue permeability. This effect has been demonstrated with adrenal cortex extract (1–3), adrenocorticotrophic hormone (1–3), and cortisone (1, 4).

Conditions of nonspecific stress imposed upon an animal by its environment are capable of effecting secretion by the adrenal gland, thus altering connective tissue permeability. Seifter, *et al.* (5), showed that adrenal cortical products endogenously released in response to the alarm reaction decreased the permeability of the synovial membrane of rabbits and antagonized the spreading effect of hyaluronidase thereon. Seifter, *et al.* (6), made a similar observation upon excised bladders of rabbits. Linderholm (7) noted decreased intradermal spread in rabbits which had been exposed to cold for fifteen or sixteen hours. Opsahl (8) observed decreased dermal spread in mice exposed to summer weather conditions. She attributes the above effect to the temperature factor; however, it is reasonable to assume that elevated atmospheric humidity was also a factor.

Hayes, Reed, and Baker (9) showed that local treatment of rat skin for four months with adrenal cortex extract caused an increase in dermal permeability at the treated site. Hayes and Baker (10) accomplished the same result after parenteral treatment with an adrenal cortex extract for thirty-seven days. One can postulate a similar increase in connective tissue permeability after prolonged exposure to stress.

Cortisone, ACTH, and adrenal cortex extract are often administered clinically over long periods of time. Conditions of stress are encountered in nature over extended periods of time. The present study was undertaken to evaluate the effect of long term stress upon dermal permeability.

Adrenalectomy has been shown to result in increased dermal permeability in mice. This effect was reported by Opsahl (11) and Winter and Flataker (2). The former worker was able to abolish not only the effect of adrenalectomy with adrenal cortex extract, but also reduced the spread to levels comparable to those obtained with the extract in intact mice, whereas the latter workers were able to abolish the effect of adrenalectomy by adding chloride to the drinking water.

EXPERIMENTAL AND RESULTS

Methods.—Connective tissue permeability was assayed by the technique of intradermally injecting albino Wistar strain rats with India ink solutions. Prior to injection the hair was clipped closely from the proposed injection sites. During the assay procedure, intact rats were anesthetized with veterinary sodium pentobarbital (6.5%) solution, adrenalectomized rats with anesthetic ether. The indicator solutions contained 17% by volume of India ink in isotonic sodium chloride solution. Injections were made both with and without hyaluronidase. The enzyme injections contained 25 Viscosity Units of hyaluronidase. Injection volume was 0.05 cc. injected with a 27-gauge needle at the rate of 0.002 cc. per second by means of a microinjector designed by Woodrow (12). Measurements were made of the length and width of the ellipsoidal area of spread forty-eight hours after injection. The area of spread was calculated by substitution in the formula for an ellipse. Measurements were made from the inner skin surface unless otherwise stated. Immediately following sacrifice, the skin was removed and pinned down to a balsa wood board with the inner skin surface upward. If a skin sample was desired for moisture determination a round section of skin 3 cm. in diameter was then removed. The section was weighed ten minutes after sacrifice and dried for eighteen hours in a hot air oven maintained at 110°.

Adrenalectomy was performed under ether anesthesia by the dorsal route. One per cent sodium chloride was added to the drinking water for two days after adrenalectomy. Purina dog chow and water were supplied *ad libitum*.

In preliminary experiments the effects of three factors upon dermal permeability were studied.

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Abstracted in part from a dissertation presented to the Graduate School of The Ohio State University by Michael M. Clay in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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Locus and Time Effect.—Variation in spread from locus to locus of the skin as well as change in spread fourteen days after injection was investigated. Locus variation should not be permitted if a single animal is to be used as its own control. Knowledge of alterations in area of spread with time is of interest in elucidation of the fate of the injected India ink particles as well as in evaluating treatments conducted with considerable intervening time between injection and measurement. Eight male rats (275–364 Gm.) were maintained in an environment in which the temperature and atmospheric humidity ranged approximately from 102° to 78° F. and 95% to 36%, respectively. These conditions were maintained throughout the experiment. The rats were divided into 2 groups of 4 rats each. Four intradermal injections of India ink and 4 of India ink-hyaluronidase solution were made on the first day (D-1) of the experiment as follows: Injections 1, 2, and 3 midway between the dorsal and ventral midlines with No. 1 over the ribs, No. 3 at the point of articulation of the hip bone, No. 2 midway between No. 1 and No. 3, and No. 4, 2.5 cm. directly ventral of No. 2. The enzyme injections were made on the right side, the ink injections on the left side. Group 1 was sacrificed on D-3 and the zone of spread measured. Group 2 measurements were made on D-14.

TABLE I.—LOCUS AND TIME EFFECTS

Group	Assay	Mean Area of Spread, mm.[1] No. 1	No. 2	No. 3	No. 4
1[a]	Enzyme	121	119	131	183
	Saline	63	61	56	74
2[b]	Enzyme	98	113	135	161
	Saline	46	46	47	89

[a] Injected on D-1, measured on D-3.
[b] Injected on D-1, measured on D-14.

The results (Table I) show that for Groups 1 and 2 the variations among Loci 1, 2, and 3 fall within narrow limits for both saline and enzyme injections. The ventral injection, No. 4, showed greater spreads than did the 3 flank injections in both Groups 1 and 2 for both ink and enzyme injections. The data presented indicate that the area of an intradermal injection decreases with the passage of time. Loci 1, 2, and 3 were selected for all further studies of dermal permeability unless otherwise stated.

Chronic Heat and Humidity Stress.—The effect of chronic heat and humidity stress upon dermal permeability was studied. Ten female rats (70–80 Gm.) were maintained for five days at 78° F. and 46% relative humidity after arrival from the animal farm. Group 1 (5 rats) was transferred to an environment subject to variable conditions of temperature and atmospheric humidity ranging from about 86° F. to 60° F. and 88% to 20%, respectively, for fourteen days. Group 2 (5 rats) was not transferred. Assays of skin permeability were made on the fourteenth day.

Table II shows that exposure for fourteen days to environmental heat and humidity excess caused decreased enzyme spread but not saline spread.

TABLE II.—EFFECT OF CHRONIC HEAT AND HUMIDITY

Assay	Mean Area of Spread, mm.[2] (± S. E.) Group 1	Group 2	P[a]
Enzyme	150 ± 18	214 ± 2	0.02
Saline	113 ± 10	117 ± 3	0.5

[a] Probability of a chance difference between the means.

Release from Stress.—The effect of a constant environment with relatively favorable conditions of temperature and atmospheric humidity upon dermal permeability of chronically heat- and humidity-stressed rats was studied. The present study was undertaken in order to determine whether prolonged heat and humidity stress will result in a rebound in permeability, and if not, whether the reduced permeability could be restored to prestress levels by maintenance in an environment of constant temperature (78° F.) and relative humidity (46%) for a protracted period of time. Five male rats (about 100 Gm.) were maintained for a period of four months under conditions of variable temperature and atmospheric humidity ranging from approximately 102° F. to 78° F. and 88% to 40%, respectively. At the end of the above period, the range of body weights was from 275–325 Gm. Experimental treatment was begun at the termination of the four-month period. Intradermal injections of India ink solution and India ink-hyaluronidase solution were made on Day 1 (D-1). The animals were maintained in the constant temperature humidity room for 22 days (from D-3 to D-24); assays were repeated on D-24.

The results in Table III show that both enzyme and saline spreads were markedly reduced by four months of stress when the values are compared with those for relatively nonstressed rats (see Table III, Group 2). Release from stress for two weeks effected significant increases in spread which did not, however, return to levels of the above relatively nonstressed rats.

Adrenalectomy.—A study was undertaken in order to determine the effect of adrenalectomy upon

TABLE III.—EFFECT OF RELEASE FROM STRESS

Mean Area of Spread, mm.[2] (± S. E.) D-1 ase[b]	D-1 sal.[c]	D-24 ase	D-24 sal.	Increase in Spread, % (D-1 vs. D-24) ase	sal.	P[d] ase	sal.	Adrenal[a] Wt., mg. D-26	Body Wt., Gm. D-1	D-26	Skin Moisture, D-26
87 ± 5	53 ± 6	133 ± 5	91 ± 15	72	58	0.01	0.05	13.2	286	300	56

[a] Average of two glands.
[b] Enzyme.
[c] Normal saline.
[d] Probability of a chance difference between the means of the D-1 vs. D-24 assays.

TABLE IV.—EFFECT OF ADRENALECTOMY

Mean Area of Spread, mm.² (± S. E.)				Increase in Spread D-21 vs. D-27, %		P[a]	Body Wt., Gm [d]	
D-21		D-27					D-21	D-29
ase[b]	sal.[c]	ase	sal.	ase	sal.	...	...	...
56 ± 15	46 ± 15	62 ± 24	34 ± 18	11	...	0.5	160	149

[a] Probability of a chance difference between the means of the D-21 vs. D-27 enzyme assays.
[b] Enzyme.
[c] Normal saline.
[d] D = Days after adrenalectomy.

the permeability of connective tissue of the skin of chronically heat-and-humidity-stressed rats. The study also served the purpose of determining whether the release of endogenous adrenal cortical hormones produced as the result of the stress of the intradermal injection procedure might have caused the reduction in skin permeability in the chronically stressed intact animals reported elsewhere in the present paper. Five female rats ranging in weight from about 70 to 80 Gm. at time of arrival from the animal farm were maintained for two weeks in an environment with constant conditions of temperature (78° F.) and relative humidity (46%). The animals were transferred to an environment subject to variations of temperature and relative humidity ranging approximately from 86° F. to 60° F. and 88% to 20%, respectively, for twenty-one days, a period constituting the period of stress. On the twenty-first day (D-21) intradermal injections of India ink solution and India ink-hyaluronidase solution were made. On D-23 adrenalectomy was performed. For two days postadrenalectomy 1% sodium chloride was added to the drinking water, followed by four days on tap water alone. On D 27, the assays were repeated. The results are shown in Table IV.

SUMMARY

1. Any change with time in the area of spread at fourteen days is toward decreased values.

2. Heat and humidity stress over a fourteen-day period effected decreased spread of intradermally injected hyaluronidase solution but not of normal saline injections. Treatment for twenty-one days effected decreased spread of both saline and enzyme injections to the degree that spreading ceased immediately after injection.

3. Maintenance under relatively favorable constant conditions of temperature and atmospheric humidity effected increases of 72 and 58 per cent for normal saline and hyaluronidase injections, respectively.

4. Adrenalectomy did not effect an increase in dermal permeability.

5. Chronic heat and humidity stress for four months did not bring about a rebound in dermal connective tissue permeability.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation of the Illinois Institute of Technology, 35 West 33rd Street, Chicago 16, Ill., seeks information on sources of supply for the following chemicals:

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n-Dodecanesulfonic acid
Neopentyl alcohol
3-Hydroxymethyl-2,4-pentanediol
Tetrachlorohydroquinone diacetate
2-Nitro-4,5-dimethylaniline
3-Pyridinesulfonamide
Glycidic acid
Naphthalene-2,1-diazo-oxide
9-Decenoic acid
Dimethyl diglycollate
Disodium methylarsinate
Stachyose
3-Hydroxykynurenine

Effects of Cortisone Acetate and Adrenocorticotrophic Hormone Upon Chronically Heat- and Humidity-Stressed Rats*

By MICHAEL M. CLAY† and JOHN W. NELSON‡

The effect of cortisone acetate and ACTH upon the dermal permeability of chronically heat- and humidity-stressed rats was studied. Treatment with cortisone and ACTH resulted in increased dermal permeability. The resulting high level of dermal permeability was not reduced by acute administration of cortisone acetate or cortisone acetate and adrenal cortex extract.

HAYES, REED, AND BAKER (1) showed that local treatment of rat skin for four months with 0.1 cc. adrenal cortex extract per day caused an increase in the area and rate of spread of a hemoglobin-hyaluronidase solution but not of a saline solution injected into the treated site. Hayes and Baker (2) also accomplished increased spread of a hyaluronidase solution in rats after treatment with 2.0 cc. of Lipo-Adrenal Extract (Upjohn) once daily for nine days and thereafter 1.0 cc. two times daily for twenty-eight days. The difference between enzyme spread in their treated animals and that observed after a single injection of adrenal cortex extract (decreased spread) was thought to be due to inhibition by the extract of the enzyme when steroids and enzymes are present simultaneously; however, with prolonged treatment structural changes are produced in the substrate which permit more rapid spreading activity by the enzyme. The change in ground substance could be a reduction in the amount.

Evidence has been presented elsewhere by the present authors in THIS JOURNAL to the effect that heat and humidity stress imposed for four months upon a group of rats did not culminate in a rise in permeability such as that noted above. If adrenal secretion were uninterrupted during the four-month period in response to the condition of stress, a rebound in dermal permeability would be expected. The absence of such a rebound could be due to inability of the chronically stressed rats to produce ACTH, refractoriness of the adrenal cortex to the action of ACTH, or refractoriness of the local tissues (dermal connective tissue) to the action of the adrenal cortex secretion. The purpose of the present study was to test the above three hypotheses.

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Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.
Abstracted in part from a dissertation presented to the Graduate School of The Ohio State University by Michael M. Clay in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Recipient of Parke, Davis & Company Fellowship in Pharmacology (1951–1952). Present address: College of Pharmacy, Columbia University.
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METHODS AND MATERIALS

The techniques employed in the present study have been described elsewhere in THIS JOURNAL. The hormones studied were cortisone acetate (a saline suspension containing 25 mg./cc.) and ACTH (1 I. U. the equivalent of 1 mg. of Standard LA-1-A). Parenteral normal saline and distilled water were used for dilution purposes and control injections

EXPERIMENTAL

Study 1.—Chronically heat- and humidity-stressed rats were treated with cortisone acetate in order to determine whether their dermal connective tissue is refractory to the action of the steroid. Twenty-five male rats (90–110 Gm.) were maintained for three months under conditions of variable temperature and humidity ranging approximately from 102° F. to 78° F. and 88% to 40% respectively. Body weights ranged from 207–280 Gm. at the end of the period of stress.

Intradermal injections of normal saline solution and hyaluronidase solution were made on the first day of the study (D-1). On D-3 the rats were divided into 4 groups and distributed as follows: Groups 1 (10 rats) and 2 (5 rats) to the constant temperature-humidity room (78° F. and 46%), and Groups 3 (5 rats) and 4 (5 rats) to the uncontrolled conditions of the animal room. Five animals were maintained per cage. Groups 1 and 3 were injected daily with a subcutaneous dose of 5 mg./100 Gm. body weight of cortisone acetate suspension for seven days. Groups 2 and 4 were given no treatment. Intradermal injections were repeated on all groups on D-11 and injection of cortisone resumed on D-13 in Groups 1 and 3 for seven more days. Skin tests were repeated on all groups on D-21.

The results of the treatment are presented in Tables I and II.

Study 2.—The minimal duration of treatment with a daily subcutaneous dose of 5 mg./100 Gm. body weight of cortisone acetate to cause increased permeability in chronically stressed rats was studied. Eighteen male rats (90–110 Gm.) were maintained for three months under conditions of variable temperature and relative humidity ranging approximately from 102° F to 78° F. and 87% to 40%,

TABLE I.—EFFECT OF PROLONGED CORTISONE TREATMENT

Group	Rats Surviving	Mean Area of Spread, mm.[a] D-1 ase[b]	D-1 sal.[c]	D-11 ase	D-11 sal.	D-21 ase	D-21 sal.	Body Wt., Gm. D-1	Body Wt., Gm. D-23	Adrenal Wt.,[a] mg. D-23
1	9	76	44	177	68	277	103	227	173	9.7
2	4	64	91	88	54	103	60	258	302	18.9
3	3	45	36	121	60	137	76	240	171	9.7
4	4	24	22	23	23	23	21	252	266	16.5

[a] Average of two glands.
[b] Hyaluronidase.
[c] Normal saline.

TABLE II.—SYNOPSIS OF RESULTS—STUDY 1

Group	Test	Per Cent Change in Spread: Within Subgroups D-1:D-11	Within Subgroups D-1:D-21	Between Subgroups D-1:D-1	Between Subgroups D-11:D-11	Between Subgroups D-21:D-21	Per Cent Change in Body Weight D-1:D-23
1	Enzyme	(+)133	(+)265	(+)18	(+)100	(+)169	(−)11
	Saline	(+) 55	(+)134	(−)52	(+) 26	(+) 72	...
2	Enzyme	(+) 38	(+) 61	Control	Control	Control	(+)19
	Saline	(−) 69	(−) 52	...	...	...	...
3	Enzyme	(+)167	(+)204	(+)88	(+)426	(+)496	(−)29
	Saline	(+) 67	(+)111	(+)64	(+)161	(+)262	...
4	Enzyme	(−) 4	(−) 4	Control	Control	Control	...
	Saline	(+) 5	(−) 5	...	...	...	(+) 6

respectively. Body weights ranged from 232–407 Gm. at the end of the period of stress.

Intradermal injections of normal saline solution and hyaluronidase solution were made on the first day of the study (D-1). On D-3 the rats were divided into 6 groups of three rats each. The animals were maintained for the duration of the study in an environment with variable conditions of temperature and relative humidity ranging approximately from 85° F. to 60° F. and 85% to 36%, respectively.

Groups 1, 2, 3, 4, and 5 received 1, 2, 3, 4, and 5 daily doses of cortisone, respectively, beginning on D-3. Group 6 was treated as described above for seven consecutive days followed by a lapse of four days; treatment was then resumed for seven days. Two days after the last injection of each group assays of skin permeability were made. In the case of Group 6 assays were made on D-11 and D-21. The results are presented in Tables III and IV.

Study 3.—Graded doses of cortisone acetate were administered to groups of rats in order to establish minimum effective doses necessary to produce increased dermal permeability. A control group was included in order to establish the effect of the drug vehicle and handling of the treated groups. Twenty-four female rats (70–80 Gm.) were maintained for two months under conditions of variable temperature and relative humidity ranging approximately from 102° F. to 78° F. and 98% to 40%, respectively. Body weights ranged from 184 to 140 Gm. at the end of the period of stress.

Intradermal injections of normal saline and hyaluronidase solution were made on the first day of the study (D-1). On D-3 the animals were transferred to the constant temperature and humidity room (78° F. and 46%) and divided into treatment groups of three rats each. The following daily subcutaneous dosages were administered, calculated on the basis of 100 Gm. body weight.

Groups 1*a*, 1*b*, and 1*c* were administered 5.0, 0.5, and 0.05 mg. cortisone acetate; Groups 2*a*, 2*b*, and 2*c* were administered 5.0, 0.5, and 0.05 mg. ACTH; Group 3*a* was administered 0.3 cc. normal saline; and Group 3*b* was given no treatment.

Submaximal doses were administered in volumes equal to those of the maximal doses by dilution with normal saline.

TABLE III.—EFFECTS OF DURATION OF TREATMENT WITH CORTISONE

Group	Mean Area of Spread, mm.[a] D-1[a] ase[d]	D-1[a] sal.[e]	D-X[b] ase	D-X[b] sal.	Body Weight, Gm. D-X	Body Weight, Gm. D-Y[c]	Adrenal, mg. D-Y[f]	H_2O in Skin Sample, % D-Y
1	88	53	187	113	326	326	13.3	61
2	77	37	214	76	302	300	11.5	60
3	95	82	301	88	334	327	11.0	62
4	111	61	321	131	325	297	11.6	60
5	93	66	338	127	330	273	8.7	58
6	119	87	441	171	303	251	6.7	57

[a] D-1, two days before first cortisone injection.
[b] D-X, two days after last cortisone injection.
[c] D-Y, four days after last cortisone injection.
[d] Hyaluronidase.
[e] Normal saline.
[f] Average of two glands.

TABLE IV.—SYNOPSIS OF RESULTS—STUDY 2

Group	Test	Per Cent Change in Spread: Within Groups D-1:D-X	Between Groups D-1:D-1	Between Groups D-X:D-X	Per Cent Change in Body Weight D-1:D-Y	Adrenal Gland Wt., mg. D-Y	Per Cent of H_2O in Skin Sample D-Y
1	Enzyme	(+)114	Control	Control	0	13.3	61
	Saline	(+)113	...	...	...	...	...
2	Enzyme	(+)178	(−)13	(+) 14	0	11.5	60
	Saline	(+)105	(−)30	(−) 49	...	...	...
3	Enzyme	(+)217	(+) 8	(+) 61	(−) 2	11.0	62
	Saline	(+) 7	(+)55	(−) 22	...	...	...
4	Enzyme	(+)198	(+)26	(+) 72	(−) 9	11.6	60
	Saline	(+)115	(+)15	(+) 16	...	...	...
5	Enzyme	(+)258	(+) 6	(+) 87	(−)17	8.7	58
	Saline	(+) 92	(+)25	(+) 12	...	...	...
6	Enzyme	(+)271	(+)35	(+)136	(−)17	6.7	57
	Saline	(+) 97	(+)64	(+)151	...	...	...

Each group except 3*b*, which was maintained in a separate cage, was kept in an individual compartment of a cage divided into four compartments. Group 3*a* was maintained in a compartment of the cage holding Group 1.

Injections were carried out in the following manner. A cage was carried from the constant temperature-humidity room to an adjoining room not similarly regulated. Each animal was weighed, then injected. The order of injection of the subgroups was randomized from day to day. These animals were returned to the constant temperature-humidity room and the remaining cage similarly handled. Group 3*b* was not handled. Treatment was continued for seven days (D-9). Skin tests were made on all groups on D-11 and treatment resumed on D-13 for seven more days. Skin tests were repeated on all groups on D-21. The results of the study are presented in Tables V and VI.

Study 4.—The nature of dermal permeability at varying time intervals after prolonged treatment of chronically stressed rats with cortisone acetate was studied. Forty-nine female rats (70–80 Gm.) were maintained under variable conditions of temperature and relative humidity ranging from about 88° F. to 60° F. and 88% to 36%, respectively, for two months. After this time body weights ranged from 215 to 150 Gm.

The animals were adrenalectomized on D-1. Drinking water was supplemented with 1% NaCl for the first two post-operative days only. During the experimental period the animals were maintained under the variable conditions of temperature and relative humidity described above.

Group 1.—Five rats were given intradermal hyaluronidase injections on D-4.

Group 2.—Thirty-seven rats beginning on D-6 were administered cortisone acetate suspension at the rate of 5 mg./100 Gm. body weight subcutaneously in single daily doses for seven days followed after a lapse of four days by a second series of seven similar daily injections. These animals were not given precortisone treatment control intradermal injections, as the possibility of renewed spread of precortisone zones had been suggested by earlier studies. The average of the spreads of the five rats of Group 1 will be considered as the pretreatment value for the cortisone treated rats of Group 2. Group 2 was in turn subdivided and intradermally injected with India-ink hyaluronidase solution at the following time intervals after the last cortisone injection: subgroup *a*—(7 rats), 90 minutes; subgroup *b*—(7 rats), 48 hours; subgroup *c*—(7 rats), 72 hours; subgroup *d*—(8 rats), 10 days; and subgroup *e*—(8 rats), 50 days.

Group 3.—Seven rats were assayed for dermal permeability established posttreatment control values.

The results of the study are presented in Table VII.

Study 5.—The eight rats of Study 4, Group 2, were studied further in order to ascertain the effect, if any, of adrenal cortical hormones upon dermal permeability of chronically stressed rats which have been further treated to bring about increased spread. Fourteen days after cessation of cortisone treatment a subcutaneous injection of 5 mg./100 Gm. body weight of cortisone acetate suspension was

TABLE V.—EFFECTS OF GRADED DOSAGE OF CORTISONE AND ACTH

Group	Mean Area of Spread, mm.[a] D-1 ase[b]	D-1 sal.[c]	D-11 ase	D-11 sal.	D-22 ase	D-22 sal.	Body Weight, Gm. D-1	Body Weight, Gm. D-24	Adrenal Weight, mg.[a] D-24	Skin H_2O % D-24
1a	121	81	228	151	316	200	175	133	10.3	58
1b	180	82	141	76	312	128	179	172	14.5	58
1c	152	67	132	53	212	115	163	174	20.4	59
2a	134	90	104	131	168	107	176	185	23.0	56
2b	118	86	149	112	218	114	166	170	29.7	59
2c	116	82	110	124	191	122	186	180	20.6	57
3a	88	50	106	82	172	101	171	176	21.3	56
3b	60	46	72	50	87	40	164	184	16.8	60

[a] Average of two glands.
[b] Hyaluronidase.
[c] Normal saline.

TABLE VI.—SYNOPSIS OF RESULTS—STUDY 3

Group	Test	Per Cent Change Spread: Within Subgroups D-1:D-11	Within Subgroups D-1:D-21	Between Subgroups D-1:D-1	Between Subgroups D-11:D-11	Between Subgroups D-21:D-21	Per Cent Change in Body Weight D-1:D-23
1a	Enzyme	+88	+161	+102	+216	+263	(−)24
	Saline	+86	+147	+ 76	+202	+400	...
1b	Enzyme	(−)22	+ 73	+200	+ 96	+259	(−) 4
	Saline	(−)10	+ 56	+ 78	+ 52	+220	...
1c	Enzyme	(−)13	+ 40	+153	+ 83	+144	+ 7
	Saline	(−)21	+ 72	+ 46	+ 6	+188	...
2a	Enzyme	(−)22	+ 25	+123	+ 44	+ 93	+ 5
	Saline	+45	+ 19	+ 96	+162	+168	...
2b	Enzyme	+26	+ 85	+ 97	+107	+151	+ 2
	Saline	+30	+ 33	+ 87	+124	+185	...
2c	Enzyme	(−) 5	+ 55	+ 93	+ 53	+120	(−) 3
	Saline	+51	+ 50	+ 78	+148	+205	...
3a	Enzyme	(−)21	+ 96	+ 47	+ 47	+ 98	+ 3
	Saline	+64	+102	+ 9	+ 64	+153	...
3b	Enzyme	+20	+ 45	...	Control	...	+ 12
	Saline	+ 9	(−) 13	...	...	...	...

made in each rat at a time which will be referred to as T_1 (initial time). An interperitoneal injection of 1 cc. of adrenal cortex extract was made in each rat at T_1 plus eighteen hours. An intradermal injection of India-ink hyaluronidase solution was given each rat, successive rats being injected at ten-minute intervals. The first rat was injected at T_1 plus twenty hours. Immediately preceding intradermal injection, an interperitoneal injection of 1 cc. of adrenal cortex extract (ACE) was made in each rat.

TABLE VII.—DURATION OF THE PERMEABILITY REBOUND

Group[a]	No. of Rats D-1	No. of Rats D-24	Mean Area of Spread Enzyme, mm.² (± S. E.)
1	5	..	66 ± 5
2a	7	7	184 ± 29
2b	7	5	310 ± 69
2c	7	7	282 ± 23
2d	8	8	405 ± 47
2e	8	8	286 ± 19
3	7	4	74

[a] Group 1—No treatment, assayed on D-4.
Group 2—a, b, c, d, and e—assayed 90 minutes, 48 hours, 72 hours, 10 days, and 50 days after last Cpd. E injection, respectively.
Group 3—No treatment, assayed on D-24.

Study 6.—Twenty of the adrenalectomized, cortisone-treated rats of Study 4, Group 2, were divided by random apportionment into three treatment subgroups, *a*, *b*, and *c*, of 6, 7, and 7 rats, respectively. Beginning forty-eight hours after the last cortisone injection intradermal injections of India-ink solution, India-ink hyaluronidase solution and India-ink hyaluronidase-adrenal cortex extract solution were made. All subgroups received injections of the same India-ink and India-ink hyaluronidase solutions. The India-ink hyaluronidase-adrenal cortex extract injections varied with the subgroups in the following manner: Subgroups *a*, *b*, and *c* were injected with India-ink hyaluronidase-adrenal cortex extract mixtures containing 0.04 cc., 0.02 cc., and 0.01 cc. of ACE/0.05 cc. injection, respectively. All intradermal injections were adjusted to contain the same percentage of ethyl alcohol as was contained in the maximal ACE concentration, that is, subgroup *a*. The ACE was mixed with the India-ink hyaluronidase solution ten minutes before the first intradermal injection of each subgroup. Members of each subgroup were given intradermal injections containing ACE at ten-minute intervals. Forty-eight hours after completion of the skin tests the zones of spread were measured from the outer skin surfaces. Intradermal injections were made on either one of both sides of the dorsal midline, approximately 2 cm. from the midline. The results are presented in Table VIII.

TABLE VIII.—EFFECT OF SIMULTANEOUS INJECTION OF ACE AND HYALURONIDASE

Group	Number of Assays	Mean Area of Spread, mm.² (± S. E.) ACE[a] + ase[b]	ase	sal.[c]
a	9	154 ± 9	151 ± 8	71 ± 5
b	11	169 ± 9	137 ± 6	82 ± 4
c	10	152 ± 15	165 ± 20	67 ± 6

[a] Adrenal cortex extract.
[b] Hyaluronidase.
[c] Normal saline.

RESULTS

Study 1.—Areas of spread of the hyaluronidase and normal saline solutions after seven and fourteen cortisone injections increased in relation to the D-1 assays of these groups as well as in relation to their nontreated controls. However, the values at all three assay times for Group 3 were lower than those of Group 1. The same observation can be made in comparing Groups 4 and 2. Consequently, continued exposure to variable environmental conditions of temperature and humidity excess counteracted the spreading of cortisone treatment on the permeability of the skin.

Study 2.—A progressive increase is generally noted in posttreatment spread for each increment of time of treatment with cortisone beginning with a single dose. Each treatment group shows an increase of spread for the posttreatment assays when compared to pretreatment levels for both enzyme and normal saline injections.

With increasing duration of treatment adrenal weight decreased, a trend which was reversed only

in Group 4. There is a similar trend toward decreasing body weight. Groups 1 and 2 showed body weights which were static during the study period. Changes in moisture content of a sample of skin were not profound enough to warrant conclusions concerning possible relationship between changes therein and changes in skin permeability.

Study 3.—Within Group 1, the D-22 zones of spread have been shown to be larger than those of D-1 and D-11; however, the D-11 values do not appear consistently greater than the D-1 values. The apparent discrepancy is clarified when the value for any particular assay of Group 1 is compared with the nontreated Group 3*b*; that is, the pretreatment spreads of Group 1, as are all of its assays, are larger than comparable assays of the control group.

Changes in spread in Group 2 essentially duplicate those of Group 1 as described above; however, when comparing the effect of cortisone acetate with that of ACTH on a dosage basis, cortisone is in general seen to be the more effective in the dosage forms administered. Zones of spread of D-22 of Group 2 do not vary according to the magnitude of the dose, nor is there a great difference in the zones of spread of Group 2 or Group 1*c* as compared with those of the saline-injected Group 1*a*. It may be postulated that much of the increased spread in Group 2 and 1*c* is due to the same mechanism which produced that of Group 3*a*; in fact, changes in body weight and adrenal weights are parallel. The increase in area of spread noted in Group 1*c*, Group 2, and Group 3*a* as compared with that of Group 3*a* may be attributed either wholly or in part to the psychic trauma of handling and injection or to the normal saline of the injections.

Values for zones of spread for D-1 of the treated animals are consistently greater than those for those of the nontreated control animals of Group 3*b*.

No conclusions can be reached concerning a possible correlation between dermal permeability and moisture content of the skin.

Study 4.—The increase in spread elicited by cortisone in chronically stressed adrenalectomized rats did not decrease the pretreatment levels after probable elimination of the cortisone which brought about the increase. Control Group 3 showed zones of spread which are approximately equal to those of Group 1, the pretreatment value.

Study 5.—The mean area of spread for the assays made four days prior to T_1 was 405 mm.^{2} ($\pm$ 47) and that for T_1 plus twenty-one hours (after cortisone and ACE treatment) was 574 mm.^{2} ($\pm$ 54).

A comparison of the means of the areas of the zones of spread of the permeability assays made four days prior to the treatment carried out in the present problem with those made after such treatment does not suggest a decrease in permeability. Indeed, the presence of circulating adrenal cortex extract and cortisone caused an increase of 40% in the area of zones of spread (<0.05).

Study 6.—Adrenal cortex extract in the concentrations used and injected simultaneously with hyaluronidase solution did not cause a decrease in the area of the zones of spread when compared with standard hyaluronidase injection solution. No effect on the area of the zones of spread from concentration to concentration of adrenal cortex extract was observed.

SUMMARY

With due cognizance of the fact that the number of animals in some groups is less than desirable it appears that the following results obtained in chronically heat- and humidity-stressed rats are justified.

1. Adrenal cortex hormones caused increased dermal permeability ("the rebound phenomenon") not only to hyaluronidase but also to normal saline solutions.

2. Dermal permeability was increased by 5.0 mg./100 Gm. body weight in a single subcutaneous dose of cortisone and further increased by succeeding daily doses.

3. Dermal permeability increased after 14 subcutaneous injections of cortisone or ACTH in dosages which are possibly within physiologic range.

4. Dermal permeability was increased by 14 daily subcutaneous injections of normal saline solution.

5. After the rebound phenomenon permeability was not decreased by the presence of adrenal cortex extract, it was increased by cortisone plus adrenal cortex extract and remained elevated fifty days after the rebound phenomenon.

6. The areas of intradermal injections of India ink made before treatment to increase dermal permeability are caused to increase by such treatment.

7. The inability of prolonged heat and humidity stress to produce a permeability rebound is not due to refractoriness of the dermal connective tissue to the secretion of the adrenal cortex as the rebound was elicited by cortisone, nor is it due to refractoriness of the adrenal cortex to ACTH stimulation, as exogenous and possibly endogenous ACTH produced the rebound. Further study with psychic stress is necessary in order to rule out the possible inability to produce ACTH in response to stress.

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The Effect of Psychic Stress Upon Dermal Permeability of Chronically Heat- and Humidity-Stressed Rats*

By MICHAEL M. CLAY† and JOHN W. NELSON‡

The effect of psychic stress upon the dermal permeability of chronically heat- and humidity-stressed rats was studied. Permeability was increased by chronic and acute psychic stress. The increase was maintained in the acutely stressed rats twenty-seven days after the stress, the last day of the study.

THE PRESENT WORKERS have reported elsewhere in THIS JOURNAL that heat and humidity stress for four months did not result in a rise in dermal permeability in rats such as that noted by Hayes and Baker (1) after administration of adrenal cortex extract for thirty-seven days, in spite of the fact that imposition of nonspecific stress is known to produce effects associated with increased level of production of corticosteroids. The present workers, in attempting to establish the basis for the above phenomenon, have shown that dermal connective tissue of chronically heat- and humidity-stressed rats can respond to exogenous cortisone and adrenocorticotrophic hormone (ACTH) by developing increased permeability. The responsiveness of the dermal connective tissue and adrenal cortex having thus been established, it is necessary to determine whether the hypophysis of the chronically heat- and humidity-stressed rat is capable of secreting sufficient ACTH to produce a rebound in dermal permeability.

The present workers have reported elsewhere in THIS JOURNAL that prolonged injection of normal saline solution in chronically stressed rats resulted in increased dermal permeability, an effect which could be due either to the injection solution or to the psychic trauma incident to the injection procedure. If the latter hypothesis is true it will be apparent that the pituitary gland of the chronically heat- and humidity-stressed rat is capable of responding to stress by secreting ACTH, although not to that stress which brought about the chronic stressed condition.

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METHODS

The techniques employed in the present study have been described in detail elsewhere in THIS JOURNAL. The permeability of connective tissue was assayed by the technique of injecting India-ink solutions intradermally in Wistar strain albino rats. Prior to injection the hair was clipped closely from the proposed injection sites. Intact rats were anesthetized with a sodium pentobarbital (6.5% solution), adrenalectomized rats with anesthetic ether during the assay procedure. The indicator solution contained 17% by volume of India ink in isotonic sodium chloride. Injections were made both with and without hyaluronidase. Each enzyme injection contained 25 viscosity units of hyaluronidase. The injection volume was 0.05 cc. injected at the rate of 0.01 cc./5 seconds by means of a microinjector. Measurements were made of the length and width of the ellipsoidal area of spread, forty-eight hours after injection, from the inner skin surface unless otherwise stated. The area of spread was calculated by substitution in the formula of an ellipse.

EXPERIMENTAL

Study 1.—A group of chronically stressed rats was injected daily with normal saline; however, the animals were not subjected to psychic trauma other than that incident to the pain of the injection. An untreated control group was similarly maintained as free from psychic trauma as possible. If the treated group showed greater spreads than the control group the increase must largely be due to the systemic effect of the injection solution. In the absence of such an effect the increase in spread noted elsewhere in THIS JOURNAL after prolonged injection of normal saline solution accompanied by psychic trauma was due to the latter factor.

Seventeen female rats (70–80 Gm.) were maintained for seven weeks under conditions of variable temperature and relative atmospheric humidity ranging approximately from 85° F. to 60° F. and 85% to 36%, respectively. Body weights ranged from about 130–190 Gm. at the end of the period of stress.

On the first day of treatment (D-1), the rats were divided into two treatment groups as follows:

Group 1.—(10 rats) 0.3 cc. normal saline/day in a single subcutaneous injection.

Group 2.—(7 rats) no treatment.

The cages of neither group were disturbed during the injection period. Each rat of Group 1 was gently removed from its cage and carefully injected, using a 27-gauge hypodermic needle. No sign of distress was discernible during the twenty-two day course of treatment. On D-22 assays of skin permeability were made.

Both groups were maintained during the course of treatment under environmental conditions of temperature and humidity similar to those of the period of stress.

Results.—The results (Table I) show that there was not a significant difference between the two treatment groups as evidenced by a probability of >0.50 for the differences between the means of both the hyaluronidase and normal saline injections. Thus, in the absence of psychic stress the injection of normal saline did not act to produce an increase in dermal permeability.

Study 2.—A group of chronically heat- and humidity-stressed rats was subjected to a severe acute psychic stress in order to determine whether the pituitary gland of such animals is capable of immediate response to stress, and in that eventuality, to learn whether the permeability of dermal connective tissue can be increased by the resulting circulating corticosteroids. Such an increase was observed elsewhere in THIS JOURNAL with a single pharmacologic dose of cortisone. In order to determine whether any observed changes in degree of spread are attributable to products of the adrenal cortex, a group of adrenalectomized heat-stressed rats was similarly subjected to acute psychic stress.

TABLE I.—EFFECT OF NORMAL SALINE INJECTIONS

Assay	Mean Area of Spread, mm.² (± S. E.) Group 1	Group 2	p[a]
Enzyme	108 ± 8	104 ± 17	>0.5
Saline	41 ± 3	43 ± 3	>0.5

[a] Probability of a chance difference between the Groups 1 and 2.

Thirteen female rats (70–80 Gm.) were maintained for seven weeks under conditions of variable temperature and relative atmospheric humidity ranging approximately from 85° F. to 60° F. and 85% to 36%, respectively. Body weights ranged from about 130–190 Gm. at the end of the period of stress.

The rats were divided into two treatment groups as follows:

Group 1.—Nine intact rats.

Group 2.—Four rats, adrenalectomized on the first day of the experiment (D-1).

The drinking water was supplemented with 1% NaCl for two days after adrenalectomy after which tap water was supplied for the balance of the treatment period.

Skin tests were carried out on D-19 on both groups. On D-21 each group was placed in a cylindrical glass jar containing water maintained at approximately 98° F. The depth of the water was such that the animals by stretching out to the fullest extent could stand with their nostrils out of the water. The intact rats were kept in the bath for two hours and fifteen minutes. The adrenalectomized rats were kept in the bath for one hour and twenty minutes at which time unfortunately, one rat drowned. The animals were gently wiped and placed in a cage maintained at about 98° F. for two hours. Permeability assays were made with hyaluronidase solutions upon removal from the heated cage.

The adrenalectomized rats were sacrificed on D-23 and the zones of spread measured. The zones of spread in the intact rats were measured from the outer skin surface on D-23 in order that a study of time-spread relationship might be made. Following that study the animals were sacrificed and the D-23 and D-19 zones of spread measured from the inner skin surface.

Results.—The mean areas of spread for Group 1 (9 survivors) were 78 mm.² (± 11) and 228 mm.² (± 20), and for Group 2 (3 survivors), 73 mm.² and 108 mm.² for the D-19 and D-21 assays, respectively.

TABLE II.—EFFECT OF ACUTE PSYCHIC STRESS

Time of Assay	Mean Area of Spread, mm.² (± S. E.) Group 1	Group 2
2 Hr. 20 Min. post-stress (D-21)	228 ± 20	108
2 Days prestress (D-19)	78 ± 11	73

The results of the study are presented in Table II. Comparison of prestress values for spread in the intact group with poststress values indicates a significant increase in spread following the bath; however, an increase was noted in the case of the adrenalectomized group although of a much smaller degree. The limited size of the latter group does not permit assumption of a mechanism for increased spread not dependent on the adrenal gland.

Study 3.—The nature of dermal permeability at varying periods of time after an acute psychic stress was studied. The nine rats which had been subjected to the stress of swimming for two hours and fifteen minutes in Study 2, were each given intradermal injections of India-ink hyaluronidase solution at the following time intervals after removal from the bath: 2 hours and 20 minutes, 24 hours, 6 days, and 27 days. Injections were begun at the indicated times with ten-minute intervals between rats. Dermal permeability had also been assayed two days prior to the acute stress treatment. Zones of spread were measured from the outer skin surface two days after injection as well as from the inner skin surface at time of sacrifice.

Results.—The results of the study (Table III) show that over the period of the study the enhanced dermal permeability which was noted soon after the rats had been removed from the bath was not decreased. There is possible significance in the 124% increase shown in the areas of the prestress skin tests within the period of two days before and twenty-seven days after the acute stress. One can thus postulate a renewal of spread in the prestress zones following the bath treatment.

DISCUSSION

Opsahl (2) has proposed that cortisone, ACTH and adrenal cortex extract inhibit the spread of hyaluronidase and saline injections by a direct blocking of hyaluronidase. Hayes and Baker (1) hold that adrenal cortex extract is inhibitory to the

TABLE III.—TIME-SPREAD RELATIONSHIP AFTER PSYCHIC STRESS

	Time of Assay				
	2 Days Prestress	2 Hr. 20 Min. Poststress	24 Hr. Poststress	6 Days Poststress	27 Days Poststress
Mean area of enzyme spread					
1[a]	78 ± 7	228 ± 21	215 ± 21	229 + 26	...
2[b]	148 ± 21	280 ± 25	206 ± 19	337 ± 66	333 ± 13

[a] Outside measurement.
[b] Inside measurement.

enzyme when both are present simultaneously. However, the present authors found that in chronically stressed rats whose dermal permeability had been increased by prolonged cortisone treatment neither pretreatment with cortisone acetate and ACE nor adrenal cortex extract injected simultaneously along with the enzyme caused a decrease in spread; indeed, in the former case a significant increase in spread was observed. Assays of skin permeability made within two to four hours after exposure to a severe psychic stress showed markedly increased spread when compared with prestress levels. Skin tests made only one and one-half hours after the last of a series of cortisone acetate injections showed increased spreads when compared with pretreatment levels. It can be assumed that in the latter two studies the level of circulating steroids was high at the time of assay.

Hayes and Baker (1) failed to obtain increased spread of saline injections after prolonged local treatment of the skin with adrenal cortex extract. In the present study with chronically stressed rats the spread of saline was shown to increase after parenteral treatment with cortisone, ACTH, and psychic stress. The question remains unanswered as to whether the increase in saline spread was a consequence of the different route of administration, or of inability of the extract to produce a phenomenon which was elicited with cortisone acetate, ACTH, and psychic stress.

Asboe-Hansen (3) explained the decreased spread of hyaluronidase injections after five-day treatment with cortisone or ACTH on the basis of a decrease in enzyme substrate quantity; whereas, Hayes and Baker (1) explain the increased spread with hyaluronidase after prolonged treatment with adrenal cortex extract on the basis of structural alterations in the substrate, probably a reduction in amount. In the present paper an increased spread was observed after two hours and fifteen minutes of acute psychic stress. However, neither a diminution nor an increase in amount of substrate within this length of time is likely. An alternative mechanism for the action of the adrenal steroids on permeability is proposed. Hyaluronidase solutions in the present study in general exhibited greater spread than did saline injections; however, in many instances the dermal spread of normal saline injections exceeded that of the enzyme in the same animal and in general the spread of the former approached that of the latter after the rebound phenomenon. Thus, postulating an antihyaluronidase action by adrenal steroids via an indirect action on the substrate, the factors which make for greater spread with the enzyme as compared to that of the saline are minimized. Adrenal steroids could cause increased permeability by decreasing the amount of substrate (after prolonged cortisone or stress treatment) or by decreasing the degree of polymerization of the substrate (after acute psychic stress).

Continued application of the environmental condition responsible for the state of chronic stress (the causal stress) failed to lead to a rebound in permeability. The fact that exogenous cortisone or endogenous corticosteroids, produced in response to exogenous ACTH or chronic or acute psychic stress, call forth the permeability rebound response would indicate that neither the connective tissue, the adrenal cortex, nor the pituitary gland is incapable of effecting the rebound response. The defect probably lies in the higher neural centers which normally operate to stimulate pituitary secretion in response to a stress such as that of excess temperature and humidity which in the present investigation served as the causal chronic stress.

The rebound to heightened permeability induced by prolonged treatment with cortisone is not merely a pharmacologic effect. A dosage level of 0.05 mg./100 Gm. body weight of cortisone or ACTH in the chronically stressed rat produced increased spread comparable to that induced by chronic psychic stress. Maximal acute psychic stress imposed by the immersion test elicited an increase in spread within a few hours comparable to that observed after fourteen daily injections of 5 mg./100 Gm. body weight of cortisone acetate. Thus, one can postulate that under natural conditions of stress the chronically stressed animal will respond in a similar manner. One can only speculate as to the nature of changes occurring under similar circumstances in other tissues of the body.

The renewal of spread of India-ink spots observed in the present study, after the rebound reaction might have a corollary in pathology. Walled-off areas of infection might under similar circumstances flare up into active infections.

The response of the individual to nonspecific stress and to adrenal cortical secretion can be modified, even reversed, by alterations in the state of the individual. Thus, the distinction between desoxycorticosterone-like hormones as the "inflammatory" hormones and cortisone-like hormones as the "anti-inflammatory" hormones is invalid. Cortisone is also an inflammatory hormone when the state of the connective tissue has been so altered as to produce that response to cortisone or to the other so-called "anti-inflammatory" hormones. Consequently it may not be necessary to assume an imbalance in the products of the adrenal cortex, specifically an increase of the mineralocorticoids to explain the mechanism for the production of many disease states. However, one can assume an increased or renewed production of a single product of the adrenal cortex and relate its

variable effects upon the altered reactivity of a local target tissue.

The action of the secretion of the adrenal cortex reverses itself with alterations in dosage level, route and form of administration or duration of treatment not only upon dermal connective tissue permeability but also upon blood cholesterol levels (4–6), histamine-induced inflammation (7), and resistance to traumatic shock (8, 9). Thus the inconsistencies noted by several workers between assayed potency of ACTH preparations and their therapeutic effects may be partially due to the different dosage levels and durations of treatment with which the various criteria of response exhibit the reversal of action.

A schematic representation of the dynamic nature of tissue permeability is presented in Fig. 1, which is intended to summarize the findings of the present investigation as well as those of other workers, coupled with conjecture.

The stages of Fig. 1 may be represented as follows:

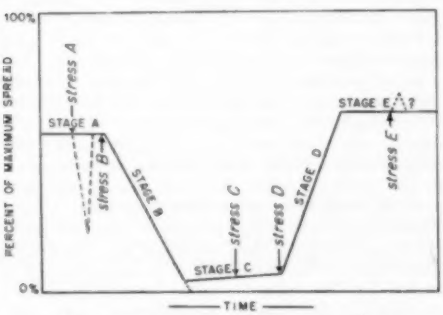

Fig. 1.—Stress permeability dynamics.

Stage A.—A state in which ACTH, ACE, cortisone, or acute stress-A cause a fall in permeability with a rapid return to or near pretreatment levels.

Stage B.—Chronic stress-B causes a decrease in permeability which might eventually reach a point where further application of stress-B causes no further decline in permeability.

Stage C.—A conditioned state in which power of adaptation to stress is inflexible, hence, minimal. Stress-B has no further effect upon permeability.

Stage D.—Administration of ACTH, ACE, or cortisone with prolonged minimal dosage or with acute maximal dosage or acute or prolonged stress-C causes a rebound in permeability.

Stage E.—A state of generalized susceptibility in which the high level of permeability unlike Stage *A* is refractory to depression by ACTH, ACE, cortisone, or acute stress-D; hence power of adaptation to stress is minimal.

The nature of the stress which can elicit the response referred to as Stage-*D* remains unclear. Psychic stress accomplished such an effect. Will other nonspecific stresses serve to produce Stage-*D* when paired against any other stress as the Stage-*C* causal factor? In the present study Stage-*C* was produced by a combination of at least two stresses. The nature of Stage-*B* *vs.* Stage-*D* relationships requires elucidation with both simple and complex stresses in both stages.

SUMMARY

The following statements may be made concerning the dermal permeability of chronically heat- and humidity-stressed rats.

1. Chronic injection of normal saline without accompanying psychic trauma did not effect increased dermal permeability such as has been obtained with psychic trauma.

2. The fact that prolonged heat and humidity stress does not cause a rebound in dermal permeability is due to the fact that the above stress has lost its ability to cause stimulation of ACTH secretion.

3. Dermal permeability was increased by acute psychic stress, an increase which was not obtained in adrenalectomized animals. The increase did not diminish twenty-seven days after exposure to the stress.

4. Nonstressed animals must be used for bioassay purposes in order to assure uniformity of results among different laboratories. When such animals are not used the nature and degree of the stress should be made known in order that a basis for comparison may be available. Ideally, specifications for standardized test animals should be prescribed.

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Blood Theophylline Concentration Following the Oral Administration of Theophylline Ethylenediamine and Theophylline Isopropanolamine*

By A. EARL VIVINO

THE DEVELOPMENT of a safe effective oral diuretic has been a goal in pharmacologic research ever since parenteral diuretics were introduced. The parenteral diuretics are known to be effective, but a wholly parenteral regimen requires frequent injections for successful diuresis and is difficult to manage with cardiac out-patients. Extensive experimental and clinical investigations indicate that oral diuretics can now give patients the advantage of effective diuresis with the convenience of oral administration. The continuous action of daily dosage results in smooth maintenance of the edema-free state. The theophylline derivatives have been used as oral diuretics but there are conflicting opinions among investigators regarding the therapeutic value of these compounds in clinical conditions for which they are prescribed. Despite the widespread interest in the theophylline compounds there is relatively little information concerning the absorption and excretion or tissue distribution of these drugs. Schack and Waxler (1) have shown that theophylline is restricted to the plasma, does not penetrate the red cell membrane, and is only slightly bound to the proteins of the blood tissues. It was suggested by these investigators that any therapeutic effect of this drug would be closely related to its level in the blood (2). Truitt, McKusick and Krantz (3) reported maximum blood levels of theophylline two and four hours after oral administration of 0.3 gram and 0.5 gram of theophylline ethylenediamine respectively; they found that satisfactory blood levels can be maintained by oral administration of theophylline derivatives.

The present study was undertaken to demonstrate the blood concentrations of theophylline at various time intervals following oral ingestion of a single dose of the drug. Two commonly used derivatives, theophylline ethylenediamine and theophylline isopropanolamine,[1] were compared.

METHODS

The theophylline blood concentrations were determined by the method of Schack and Waxler (1). The method as adopted for this study offers a precision of ±3% at levels of 100 μg. of theophylline per 100 ml. of blood. The study was based on 1,600 determinations performed on blood samples obtained from 25 normal persons between the ages of 20 and 28 years. A preliminary blood sample was obtained for the blank determination. Blood was then drawn at the following time intervals following administration of drug: 15 minutes, 30 minutes, 45 minutes, 60 minutes, 1½ hours, 2 hours, 2½ hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, and 10 hours. All determinations were performed in duplicate. The same normal persons were used for study after the oral administration of theophylline ethylenediamine and theophylline isopropanolamine. In preparation for the determinations, the individuals under study had been on no medication for one week prior to the study. Coffee, tea, and cola drinks were omitted from the diet during this investigation. Thirty days were allowed to lapse between the study of the two drugs. The evaluation was based on a single dose of the theophylline preparations. Uncoated 0.2-Gm. tablets of the preparation, each containing 0.160 Gm. of theophylline, were used. The determinations made measured the theophylline blood levels of both theophylline derivatives.

RESULTS

Table I shows the average theophylline blood level after oral administration of theophylline ethyl-

TABLE I.—AVERAGE THEOPHYLLINE BLOOD LEVELS

Time after Oral Administration, min.	Micrograms per cent of Blood: Theophylline Ethylenediamine	Theophylline Isopropanolamine
0	0	0
15	44.28	81.92
30	134.05	201.20
45	192.72	255.84
60	220.96	318.24
90	215.56	346.60
120	217.08	346.44
150	216.64	340.56
180	215.56	320.84
240	199.52	309.52
300	200.04	300.60
360	168.80	202.84
420	144.20	174.80
480	117.72	130.32
540	60.80	89.60
600	5.20	49.72

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[1] Theophylline isopropanolamine (Theopropanol®) was obtained from the National Drug Company Philadelphia 44, Pa.

enediamine and theophylline isopropanolamine at the various time intervals after administration. A high blood level was noted after the use of theophylline isopropanolamine at each time interval. At the end of ten hours theophylline was still noted in appreciable quantities in the blood after the administration of theophylline isopropanolamine, while only a few normal individuals showed small blood concentrations of theophylline ten hours after the administration of theophylline ethylenediamine. Both drugs appeared in the blood fifteen minutes following ingestion and the concentrations rose rapidly to a maximum in one hour. A nearly constant concentration was then maintained for four hours followed by a gradual decrease in blood level.

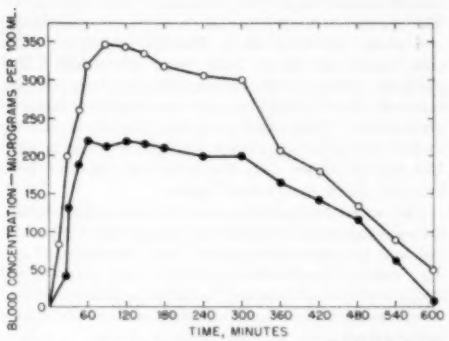

Fig. 1.—Blood concentrations of theophylline following oral administration of theophylline-ethylene diamine and theophylline-isopropanolamine. O—O, theophylline-isopropanolamine. ●—●, theophylline ethylenediamine.

DISCUSSION

Theophylline compounds and related xanthine derivatives are used extensively, but their effectiveness as diuretics is questioned by some clinical observers. The determination of the theophylline blood level can now be utilized in addition to the clinical response in more scientific regulation of theophylline therapy. The development of a method for determining blood theophylline concentration by Truitt, Carr, Bubert, and Krantz (4) and by Schack and Waxler (1) makes possible a comparison of the rates of absorption and durations of action of various theophylline derivatives. Theophylline is only slightly soluble in water and is therefore employed in practice in the form of "double salts." Theophylline ethylenediamine (aminophylline) and theophylline isopropanolamine (Theopropanol®) are such double salts and are widely used in clinical practice. Ethylenediamine and isopropanolamine added to the theophylline radical increases the solubility and absorption of theophylline and enhances its therapeutic action. Our studies of both theophylline derivatives show adequate and prolonged blood levels of theophylline in normal individuals after the oral administration of 0.2 Gm. of either compound. A more rapid rise in blood concentration and higher and more prolonged blood levels were noted after administration of theophylline isopropanolamine than after theophylline ethylenediamine. These differences are probably related to the greater solubility of theophylline isopropanolamine.

SUMMARY

1. Theophylline ethylenediamine and theophylline isopropanolamine were given to 25 normal subjects and theophylline blood concentrations were determined at various time intervals for ten hours after oral administration of 0.2 gram.

2. Theophylline appeared in the blood fifteen minutes after ingestion of either drug and there was a rapid rise in concentration to a maximum at one hour. A high level was sustained for four hours followed by gradual decrease in blood concentration over the next five hours.

3. A more rapid rise in blood concentration and higher and more prolonged blood levels were noted after administration of theophylline isopropanolamine than after theophylline ethylenediamine.

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Chemical Investigation of *Vaccinium myrtillus* L.*

By EGIL RAMSTAD

The huckleberry shrub has been subjected to chemical investigation, and several constituents were isolated among which oleanolic and ursolic acids, β-amyrin, myrtillol, and nonacosane were identified. Several fat acids, plant acids, tannins, and sugars were also identified. Hydroquinone, but not arbutin, could be detected.

HUCKLEBERRY LEAVES have enjoyed a wide variety of uses since antiquity (14) in Europe, and the *Vaccinium arboreum* March has been used in North America in diarrhea, abscesses in the mouth, and as an astringent. The literature suggests that it has been used to treat psoriasis (15) and other skin affections (16). It has been used in the treatment of coughing, vomiting, colics, atony of the bladder, chronic catarrh, mucosal inflammation, and burns. It has served in the form of lotions and cataplasms for eye infections. The leaves have been used quite frequently as a tea substitute (17). In Russia it is known as Caucasian tea or Kutai tea (16).

One of the most common uses of huckleberry leaves in folk medicine is that of an antidiabetic. Allen (18) isolated an amorphous brown fraction from huckleberry leaves and claimed that it lowered the blood sugar level. However, the huckleberry plant has been the object of very scanty chemical investigation.

It is claimed in several references on pharmacognosy that the drug contains arbutin. This claim does not seem to be based upon chemical investigation of the plant but rather inferred by virtue of a close botanical relationship of the plant to other arbutin-bearing plants. We have not been able to substantiate this claim. Also the histochemical reaction with ferrous sulfate as reported by Tunmann (20) for arbutin is negative with huckleberry leaves. The gross composition of the leaves has recently been reported (19) to be 3.14 per cent petroleum ether extractable, 0.85 per cent ether extractable, 0.11 per cent chloroform extractable; 7.81 per cent extractable with absolute alcohol, 4.3 per cent extractable with cold water, 6.22 per cent with hot water, 4.33 per cent extractable with 1 per cent HCl.

The examination of some of the constituents of huckleberry leaves is reported in this paper. However, investigations on the alleged antidiabetic principle have not been included.

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EXPERIMENTAL

Stabilization and Extraction.—Ten kilograms of fresh huckleberry shrub were stabilized with boiling alcohol in the presence of an excess of calcium carbonate, according to the method of Bourquelot (1). The stabilized material was dried in air, powdered, and then extracted in a Soxhlet apparatus with ether until no more color was given off. The alcoholic extract from the stabilization was evaporated *in vacuo* to dryness and the residue extracted with ether. The combined ethereal extracts were shaken out in a separatory funnel three times with 150 ml. of water and the ethereal extracts were brought down to dryness (lipids).

The ether-extracted plant material was subsequently extracted two times with boiling 80% alcohol and the material pressed in a hydraulic press. The combined alcoholic extracts were evaporated under reduced pressure of a water aspirator pump. The brown, brittle residue weighed 470 Gm. (alcoholic extract).

Ether Extract.—*Separation.*—The lipid fraction was mixed with 300 Gm. of anhydrous sodium sulfate, and extracted in a Soxhlet apparatus with low boiling petroleum ether (b. p. 30–60°) for twelve hours. The petroleum ether extract was evaporated to dryness. Weight: 103 Gm. (3, petroleum ether extract).

The residue in the Soxhlet apparatus was then extracted with ether for four hours. A part of the residue went into solution [1, oleanolic acid], while the remaining material required extraction for a period of several days to pass into the solvent flask, in which it deposited from the solution [2, myrtilloresene]. The latter was filtered off and weighed 6 Gm. The ethereal solution was combined with the above solution of oleanolic acid (residue 4.3 Gm. [1]).

Oleanic Acid.—Fraction [1], which showed a deep green color, was purified by precipitation from an ethereal solution with ten times the volume of petroleum ether, then recrystallized four times, each time from 300 ml. of alcohol. The melting point rose from 213–215° to 249–252°. After prolonged extraction of the substance with petroleum ether and recrystallization from acetone, the melting point was 296–298° and could not be increased by crystallization of the compound from alcohol or acetone.

The acetylated product, 0.3 Gm. (see below), was saponified with 1 *N* alcoholic KOH, the solution evaporated and the acidified residue extracted with ether. The residue from the ether extract was recrystallized from alcohol. It melted at 305–307°. (M. p. of oleanolic acid: 306–308° (2, 3), $[\alpha]_D^{20} = +77.6°$ (50.3 mg.—5.00 ml.—chloroform) (Winterstein, *et al.* (4): $[\alpha]_D^{15} = +76.5°$).

The compound is readily soluble in chloroform and in acetone, sparingly soluble in ether and in cold alcohol, insoluble in petroleum ether. It dissolves in 50% aqueous potassium hydroxide. It dissolves in concentrated sulfuric acid with brown color and gives a color change of red through purple, purplish blue, blue, and finally green in the Liebermann-Salkowski sterol test (5). The molecular weight, determined according to Rast's method (6) with camphor (m. p. 178°) was found to be 462, and determined by titration in alcoholic solution with 0.1 *N* NaOH (phenolphthalein) gave the equivalence of 451 for a monobasic acid ($C_{30}H_{48}O_3$—mol. wt. 456.7).

Acetate.—One gram of the impure substance (m. p. 249–252°) was refluxed with 50 ml. of acetic anhydride and 30 ml. of pyridine for two hours. Crystals separated out upon cooling and, after recrystallizations from alcohol and from acetone, had a m. p. of 261–263°. Markley, *et al.* (6), reported a melting point of 264.5–265.5° for the acetate of oleanolic acid. Upon saponification of the acetate and crystallization as indicated above, oleanolic acid with a m. p. of 305–307° was obtained.

Myrtilloresene.—Under the microscope, substance [2] appeared as spheroidal crystal aggregates when deposited from boiling ether during the extraction. It was sparingly soluble in alcohol but readily soluble in chloroform. When crystallized from 95% alcohol it formed fine, curved, interlaced needles. The substance had a brownish color which could not be removed by repeated crystallization from alcohol, by boiling with active charcoal, or by chromatographic adsorption on aluminum oxide, etc. It is, therefore, deducted that the substance possesses a slightly brownish color of its own. The melting point could not be raised above 83°. Its sharp melting point is another indication of purity. We name this compound *Myrtilloresene.* It has not, as yet, been investigated.

No acetate was obtained upon acetylation. The recovered material from acetylation possessed the same melting point and no depression was noticed in admixture of the substance with the starting material.

When tested for sterol by the method of Liebermann-Salkowski a color change from dirty red through dirty violet to blue resulted.

Separation of the Petroleum Ether Fraction.—Fraction [3] was dissolved in 500 ml. of ether and shaken out repeatedly with 150 ml. of a 20% potassium hydroxide solution, 50 ml. each time. Fine, sharp needles separated out in abundance at the interface between the brown aqueous layer and the black-green ether layer. The crystals were isolated by filtering the whole nixture through a course glass sinter funnel [3.1 ursolic acid].

The aqueous layer [3.2] of the filtrate was shaken repeatedly with ether and all ethereal extracts combined [3.3]. The aqueous layer [3.2] was acidified with sulfuric acid. A heavy, black precipitate separated out. The precipitate was extracted with dry ether. A part went into solution [3.21] while a greater portion remained undissolved [3.22]. The undissolved fraction consisted mainly of chlorophyllins and was discarded.

The ethereal solution [3.21] was concentrated to dryness. As evidenced from tests with 20% KOH, needles formed which had the characteristics of sodium ursolate. Another part of the residue was subjected to vacuum sublimation. Crystals were deposited on the receiver [3.221 hydroquinone]. The greater part of the residue was dissolved in 50 ml. of ether and precipitated out with 1,000 ml. of petroleum ether. The precipitate (9.2 Gm.) was still green [3.222, resinous acids].

The ethereal solution of the neutral lipids [3.3], was concentrated to dryness. It formed a semisolid, dark brown mass and contained crystals (23 Gm.). It was saponified by refluxing with 200 ml. of 1 *N* alcoholic KOH for two hours. The mixture was concentrated to about 75 ml., then diluted with 200 ml. of water and shaken out repeatedly with ether. The combined ethereal fractions were washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness forming an orange red smear [3.31 unsaponifiable: 9.6 Gm.]. The aqueous layer was acidified with sulfuric acid and shaken out with ether. The ethereal fractions were washed with water and evaporated to dryness [3.32, fatty acid fraction: 14 Gm.].

The unsaponifiable fraction [3.31] was dissolved n boiling ether. Upon cooling the solution deposited small crystals in such a quantity that the whole mixture became semisolid. The crystals were filtered off by light suction in a sinter glass filter and washed with a small quantity of ice-cold ether [3.311, nonacosane].

The mother liquors from the preparation of nonacosane were evaporated to dryness, the residue dissolved in three parts of amyl alcohol, and the solution left at −10°. After two days long needles had settled out and were filtered off [3.312, β-amyrin].

The filtrate was left for two weeks at about −30°. Crystals separated out and were isolated by pouring off the liquid and washing with a small amount of amyl alcohol [3.313, myrtillol].

The final mother liquid, containing carotenoids and other compounds, was not investigated further.

Hydroquinone.—The prismatic crystals of the sublimate dissolved in water and gave dark color with ferric chloride solution. The solution reduced ammoniacal silver nitrate. In admixture with pure hydroquinone in sealed melting tube, it melted at 168° (pure hydroquinone 169°).

Ursolic Acid.—The crystals [3.1] were suspended in ether and the mixture acidified with concentrated hydrochloric acid. After evaporation of the ether the amorphous residue was recrystallized three times from alcohol, yielding a substance of the melting point of 277–279°. The substance obtained after hydrolysis of the acetate (see below) had m. p. 283–284° (Sando (7) 282.5–284°). $[\alpha]_D^{20} = +61.0°$ (45.0 mg.—5.00 ml. of methanolic KOH) (Rowe, Orr, Uhl, and Parks: +62.1°). As the substance is not soluble enough in camphor to permit molecular weight determination according to the Rast method, the compound was dissolved in an excess of alcoholic 0.1 *N* KOH and back-titrated with 0.1 *N* HCl by use of phenolphthalein as indicator. Equivalence: 449 (0.2315 Gm.—5.15 ml. of 0.1 *N* KOH) (theory 456).

Acetate.—The substance, 0.5 Gm., was refluxed for one hour with 5 ml. of acetic anhydride and 3 ml. of pyridine. Upon evaporation of a greater

part of the solvent long needles formed. After repeated recrystallization from alcohol the acetate showed constant m. p. of 289–290°. (Sando (7): 289–290°). Deacetylation of this product by refluxing with 10% alcoholic KOH for two hours, acidification and extraction with ether gave a substance, which, after recrystallization, melted at 283–284°.

Ursolic acid dissolved in sulfuric acid with yellow color. The Liebermann-Burchard sterol reaction (8) was positive (red, violet, blue, green), while the Hager-Salkowski's reaction (9) was negative. Unlike cholesterol, ursolic acid does not give the Winogradoff reaction until after heating, nor does it form a precipitate with digitonin from alcoholic solutions.

Fatty Acids.—*Separation.*—Fourteen grams of the liquid fatty acid fraction [3.32] was dissolved in aqueous potassium hydroxide and the solution neutralized with acetic acid, then diluted with water to 300 ml. The solution was heated and a 2% lead acetate solution was added in excess. The precipitate was separated and refluxed with 100 ml. of ether and placed in an icebox for twenty-four hours. The insoluble fraction was separated and treated with boiling ether once more. The ether-insoluble lead salts and the ether-soluble salts were separately decomposed with HCl and the free acids taken up with ether. The liquid acids [3.321] recovered from the ether-soluble salts constituted 68% of the acid mixture; the solid acids constituted fraction [3.322].

Oxidation of the Unsaturated Acids with Permanganate.—Thirty grams of the liquid fatty acids [3.321] was dissolved in an excess of potassium hydroxide solution, diluted to 300 ml. and oxidized at 0° with 300 ml. of a 1.5% potassium permanganate solution. Excess of manganate oxides was eliminated by adding sodium sulfite and sulfuric acid. The hydroxy acids were filtered off, washed, dried, and extracted in a Soxhlet extraction apparatus first with petroleum ether, then for two days with ether. The ether-soluble acid was recrystallized three times from alcohol, m. p. 134° (dihydroxystearic acid from *oleic acid*). No depression of the melting point occurred in admixture with authentic dihydroxystearic acid. The ether insoluble part of the oxidized acids was crystallized from a mixture of alcohol and acetone, then three times from 50% alcohol, m. p. 172° (tetrahydroxystearic acid from *linolic acid*). Mixture of the acid with authentic tetrahydroxystearic acid had the same melting point.

Separation of the Solid Acids.—The solid acids [3.322] were esterified by refluxing with absolute methyl alcohol in the presence of HCl. The esterified acids were isolated by diluting the alcoholic solution with water and shaking out with petroleum ether. After washing and drying over sodium sulfate, the solvent was distilled off and the residue (3.9 Gm.) fractionated *in vacuo* at 15 mm. Hg into three portions: (*I*) 2.1 Gm. at 194–196°, (*II*) 1.2 Gm. at 196–198°, (III) residue 0.4 Gm. Saponification value for fraction *I* was 206 (theory for methyl *palmitate*, 207.5), for fraction *II* it was 191 (theory for methyl *stearate* 187.9).

Nonacosane.—Fraction [3.311] was recrystallized from a small volume of ether at −30°. The crystals melted at 62–64° (nonacosane: 63.5° (10)). Further purification was attempted by crystallization from alcohol, in which it is quite sparingly soluble at low temperatures and from which it crystallizes in fine curved crystal needles. Traces of a yellow substance were strongly associated with the nonacosane. However, the color could be separated by acetylating the material (acetic anhydride-pyridine), followed by recrystallization from alcohol.

The compound gave no reaction for aldehydes or ketones. Nor did it form an acetyl derivative; for the acetylated product had the same melting point as the starting material and gave no depression of the melting point in admixture with the initial material.

Molecular weight, according to Rast method: 406 (45.1 mg.—0.4886 mg.—Δ9.0°) (theory for $C_{29}H_{60}$: 408).

β-Amyrin.—The fraction [3.312] formed long fine needles. Two recrystallizations from alcohol furnished a colorless substance. Melting point 195–196° (Ruzicka (11): 197–197.5°). $[\alpha]_D^{19} = +79.9°$ (38.3 mg.—2.00 ml. of chloroform).

The Liebermann-Salkowski test furnished a cherry red, stable coloration. Gradually a fluorescence developed in the solution.

Acetate.—Two grams of the substance was refluxed for two hours with 50 ml. of acetic acid. The solution, which turned bluish green, deposited overnight big, heavy needles readily soluble in boiling, sparingly in cold acetic anhydride. The acetate is very sparingly soluble in alcohol, but can be obtained in pure form from this solvent as hexagonal plates. When dissolved in chloroform it crystallized out in silky needles on the addition of alcohol. Melting point 236°. $[\alpha]_D^{18} = +78.5°$ (55.4 mg.—5.00 ml. of chloroform) (Ruzicka (11): m. p. 236°, $[\alpha]_D^{17} = +81.4°$).

Myrtillol.—Fraction [3.312] was recrystallized from alcohol. The m. p. was 239–240° and the $[\alpha]_D^{19} = +89.1°$ (201.9 mg.—10.00 ml. of chloroform) and did not change upon further recrystallizations.

Acetate.—Acetylation carried out by boiling the substance with acetic anhydride in the presence of pyridine furnished a substance which upon recrystallization from alcohol had a m. p. of 202–203° and $[\alpha]_D^{19} = +110.8°$ (17.5 mg.—2.00 ml. of chloroform).

The compound gives color reactions for sterols and precipitates with digitonin. Its constants do not correspond to those of any known sterol as far as could be found. The compound, which is designated as *Myrtillol*, has not yet been investigated further.

Hydro-alcoholic Extract.—*Separation with Lead Acetate.*—The previously defatted alcoholic extract was dissolved in three times its weight of water. Some material was insoluble and was filtered off. The clear solution was precipitated with 10% lead acetate solution until only very little precipitate formed when further quantities of the precipitant were added. The voluminous yellow-brown precipitate was filtered off, washed with lead subacetate wash water, then suspended in water and decomposed with H_2S until excess of the latter was present. The lead sulfide was separated by filtration. The filtrate contained acidic substances. The acidic solution was passed slowly through a column of Amberlite IR-4B. The effluent was brown and astringent [4.1, tannin fraction]. The ion exchange

resin was eluated with 2% sodium hydroxide solution. The eluate was then passed through Amberlite IR-120 for generating the free acids [4.2].

Tannins.—The fraction [4.1] was evaporated to dryness *in vacuo* on a steam bath. The fraction gave the following reactions: (*a*) it was precipitated completely by lead subacetate; (*b*) it gave, in high dilution, a dirty green color with ferric chloride—a color that changed to dirty violet upon addition of sodium acetate; (*c*) it reduced Fehling solution upon boiling; (*d*) it is salted out by high concentrations of ammonium sulfate; (*e*) it gave a precipitate with a 2% solution of antipyrine; (*f*) when a 0.5% solution of the tannin fraction was boiled with 4% formaldehyde and 2% hydrochloric acid for one-half hour, and the precipitate which formed was filtered off, the filtrate did not give any coloration with ferric chloride plus sodium acetate (12) (catechin tannins only); and (*g*) when an aqueous solution of the tannin was heated with hydrochloric acid, a red precipitate ensued; the filtrate was shaken with ether and the ethereal layer shaken with a dilute aqueous solution of ferric chloride. No color developed (catechin tannins only).

The tannin fraction was chromatographed on Whatman paper No. 1. The upper layer from a mixture of butanol 4, acetic acid 1, water 5 served as irrigation fluid. Ferric chloride solution (0.2%) served as developer. Although a faint dark line developed all the way from the point of application of the tannin down to R_f of 0.75, 4 points of gravity of the dark green color were evident. These spots possessed the R_f values of 0.08, 0.33, 0.41, and 0.65. The spot of 0.08 was darker than the others and color developed much faster with $FeCl_3$. Upon standing in air for a long time, all the spots changed gradually to a black color.

When purified by extraction of the aqueous solution with ethyl acetate, the tannin fraction gave identical reactions.

Citric, Malic, and Succinic Acids.—Fraction [4.2] was chromatographed on Whatman paper No. 1 with two different solvent mixtures: (*I*) butanol 4, acetic acid 1, water 5 (upper layer), and (*II*) phenol 3, water 1, 90% formic acid 1 (upper layer). The acid spots on the chromatograms were developed with (*a*) 0.5% alcoholic solution of mercurochrome, which makes the acid spots appear in yellow color on a red background; viewed in ultraviolet light, the spots appear dark purple on yellow background; and with (*b*) 0.04% bromcresolgreen, which makes the spots stand out yellow on a blue background.

The following R_f values were obtained with Solvent *I:* 0.42 (citric acid), 0.52 (malic acid), and 0.75 (succinic acid); with Solvent *II:* 0.26 (citric acid), 0.43 (malic acid), and 0.65 (succinic acid). Authentic samples of the three identified acids were mixed with the crude acid fraction under investigation and the mixture chromatographed as above. No additional spots formed.

Judged from the area occupied by the spots on the chromatogram, the ratio of the three acids is approximately: 2 (citric acid):1 (malic acid):3 (succinic acid).

Glucose and Fructose.—The mother liquor from the preparation of sucrose (described below) was chromatographed with the above mixture of butanol, acetic acid, and water. The R_f values showed the presence of glucose (R_f = 0.24), and fructose (R_f = 0.28). Controls were run with pure sugars. The size of the spots and the weak coloration produced by heating to 105° for ten minutes of the chromatograms with 2% aniline hydrogen-phthalate, indicate that only traces of these free sugars are present.

Sucrose.—The glucide solution was deleaded with hydrogen sulfide, the mixture filtered, and the filtrate concentrated to 150 ml. to which 750 ml. absolute alcohol were added. After two months in a refrigerator, the heavy brownish crystals that had formed, were separated. The crystals were dissolved in boiling 90% alcohol, and the solution decolorized by boiling with activated charcoal. The crystals melted at about 180° with decomposition, had $[\alpha]_D^{20}$ = +65.9° (water), did not reduce Fehling solution until after being hydrolyzed by boiling with hydrochloric acid. The sugar was chromatographed with butanol 4, acetic acid 1, water 5 as the irrigation fluid (upper phase). When the chromatogram was treated with invertin according to the method of Bliss and Ramstad (13) for detection of glycosides and developing of the spot with aniline hydrogen-phthalate, a spot formed at R_f value of 0.17. When the test was run with pure sucrose added, only one spot developed. Chromatography of the hydrolyzed sugar gave two spots, R_f:0.24 (glucose) and 0.28 (fructose).

Search for Arbutin.—No crystalline material could be obtained by shaking out the glucide fraction with ethyl acetate. A part of the glucide fraction was refluxed for five minutes with 1% hydrochloride acid, the mixture shaken out repeatedly with ether, and the ether evaporated. A very small residue resulted, but it gave no reaction for hydroquinone. Arbutin also failed to show up on chromatograms prepared and treated according to Bliss's and Ramstad's (13) micromethod for detection of glycosides. When a small quantity of arbutin was mixed with the glucide fraction, arbutin showed up easily on the chromatogram. No other α- or β-glucoside could be detected.

SUMMARY

The stabilized fresh shrub of *Vaccinium myrtillus* L. was subjected to chemical analysis. The following constituents were identified:

In the Ether Extract.—oleanolic acid, myrtilloresene, hydroquinone, ursolic acid, resinous acids, oleic acid, linolic acid, palmitic acid, stearic acid, nonacosane, β-amyrin, and myrtillol.

In the Hydro-alcoholic Extract.—malic acid, succinic acid, citric acid, four tannins of the catechin type, glucose, fructose, and sucrose.

Contrary to listings in textbooks, no arbutin could be found.

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Volumetric Determination of Quaternary Ammonium Compounds and Certain Tertiary Amines*

By E. D. CARKHUFF and WM. F. BOYD

A volumetric method employing anionic surface-active agents for the quantitative determination for most quaternary and certain type amines is presented. Concentrations of Ceepryn® Chloride and Bentyl® Hydrochloride in the order of less than 1:4000 are readily titrated. The determination by this method of several similar compounds is discussed. The method employs sodium lauryl sulfate, U. S. P. or dioctyl sodium sulfosuccinate, N. F. as the titrant with the determinations being carried out in the presence of dilute sulfuric acid and chloroform, using dimethylaminoazobenzene as the indicator. A wide variety of pharmaceutical preparations, such as tablets, ointments, candy base medication, and liquids, may be assayed by the procedure outlined.

DUE TO THE relatively small amounts of certain quaternary ammonium compounds and tertiary amines required for efficacious medication, their analyses involve difficult and often unsatisfactory procedures. Various procedures have been designed and are noteworthy contributions (1–5). These procedures often require larger quantities of samples than are convenient to handle, and the interference caused by various constituents of pharmaceutical preparations either nullifies their use or requires the use of large correction factors.

The method presented is surprisingly free from interference and possesses the distinct advantage of being sensitive and accurate in low concentrations. It has been found to be readily adaptable for routine assay work and to give satisfactory results in inexperienced hands. The end point of the titration is very sharp and is reproducible to less than 0.1 ml. As a general rule, either sodium lauryl sulfate, U. S. P. or dioctyl sodium sulfosuccinate, N. F. may be employed interchangeably as the titrant.

The method has been shown to be applicable to the analysis of Ceepryn® Chloride,[1] Bentyl® Hydrochloride,[1] β-diethylaminoethyl fencholate hydrochloride, and compounds of a similar nature For purposes of presentation, the analysis of one compound will be described in detail.

METHOD

Reagents.—*Sodium Lauryl Sulfate Solution.*—Dissolve 1.2 Gm. of sodium lauryl sulfate, U. S. P. in sufficient distilled water to make 1,000 ml. *Methyl Yellow (p-dimethylaminoazobenzene) Indicator.*—Prepare a 0.01% alcoholic solution. *β-Diethylaminoethyl 1-cyclohexyl cyclohexanecarboxylate Hydrochloride (Bentyl® Hydrochloride) Solution.*—Weigh accurately 100 mg. of Bentyl® Hydrochloride and dissolve in sufficient distilled water to make 100.0 ml. *Chloroform, U. S. P.—Diluted Sulfuric Acid, N. F.*

Procedure.—Standardize the sodium lauryl sulfate solution as follows: Place into a 100-ml. glass-stoppered graduated cylinder 20.0 ml. (20.0 mg.) of Bentyl® Hydrochloride solution, 10 ml. of distilled water, 5 ml. of diluted sulfuric acid, 1 ml. of methyl yellow indicator solution, and 20 ml. of chloroform. Stopper and shake the cylinder vigor-

* Received August 21, 1953, from the Research Laboratories, The Wm. S. Merrell Company, Cincinnati, Ohio.
Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.

[1] Ceepryn® and Bentyl® are trademarks of The Wm. S. Merrell Company for cetylpyridinium chloride, and β-diethylaminoethyl 1-cyclohexyl cyclohexanecarboxylate hydrochloride (dicyclomine) hydrochloride, respectively.

ously. It will be noted that the chloroform solution develops a bright yellow color while the aqueous phase becomes clear and colorless. Titrate the liquids in the cylinder with sodium lauryl sulfate solution, shaking thoroughly after each addition until the chloroform layer develops the first orange tint. Record the buret reading. Some compounds, due to their surface activity, may emulsify at the beginning of the titration. Should this occur, proceed with the titration. The emulsion will break more readily and finally disappear at the end point.

A more precise end point may be obtained if the color of the drop of chloroform that appears on the surface of the aqueous phase is used. In some instances, the drop does not form; thus, it cannot always be used for the end point.

For compounds other than Bentyl® Hydrochloride, the same procedure is used for the standardization of the sodium lauryl sulfate, employing that compound which is undergoing analysis.

Preparation of the Sample.—The preparation of the sample is usually an individual consideration. Generally, ampul contents may be diluted to suitable concentrations and assayed forthwith.

Tablets and candy base medications are weighed, powdered, and a suitable amount accurately weighed out, transferred to a cylinder, and assayed. Some extraction procedure may be necessary to free the active constituent from insoluble carriers or diluents.

Ointments are generally accurately weighed out, transferred to the cylinder, and titrated.

Assay of the Sample.—Transfer quantitatively a sample equivalent to 10–25 mg. of Bentyl® Hydrochloride to a 100-ml. glass-stoppered graduated cylinder. Add reagents in the amounts specified in the standardization of sodium lauryl sulfate, and proceed with the titration to the first orange tint of the chloroform. The results may be calculated as follows:

$$\frac{A}{B} \times C \times D = \text{mg. in product,}$$

where A is the ml. of sodium lauryl sulfate solution required to titrate the sample; B, the ml. of sodium lauryl sulfate solution required to titrate the standard; C, the mg. of Bentyl® Hydrochloride used for standardization; and D, the dilutions used for assay.

DISCUSSION

Upon prolonged standing, the sodium lauryl sulfate solution will develop a precipitate which, apparently, has no ill effect upon the titration qualities of the solution. Even so, the precaution of restandardization prior to use should be observed.

The method is equally effective whether the Bentyl® Hydrochloride is dissolved in water or in chloroform. Thus, if during the preparation of the sample, an extraction procedure is found essential, the final solvent may be either chloroform or water. Aqueous solutions containing about 10 mg. of Bentyl® Hydrochloride per 50 ml. may be used. Chloroform solutions should not exceed 30 ml. and contain substantially not less than 10 mg. of active material. The method is effective in greater dilutions; however, the titration volume reaches a point where the accuracy of the assay is difficult to maintain unless special precautions and apparatus are used.

Several chlorinated solvents may be used in the method. Chloroform appears to be the most widely adaptable due largely to its unusual solvent powers on highly ionized compounds. Methylene chloride may be substituted in many instances with excellent results. Ethylene dichloride and carbon tetrachloride are usable but they fail to give as satisfactory end points as the aforementioned solvents.

The reaction between sodium lauryl sulfate and Bentyl® Hydrochloride appears to be stoichiometric. Known amounts of Bentyl® Hydrochloride were titrated with sodium lauryl sulfate and the resultant data when plotted showed a straight line function.

Further support of this view is given by the investigations of Jones (6) and Epton (7) in which they observed that the "sulfonate" will combine quantitatively with cationic compounds. It is postulated that the reaction involved in the outlined procedure is the formation of a stable complex between the compound and titrant. The titrant, in excess of that required for complex formation, is extracted into the chloroform layer as lauryl hydrogen sulfate and reacts with the methyl yellow to produce the color characteristic of the end point.

Acids other than sulfuric, highly ionized surface-active agents, and water-soluble solvents generally render the method inoperative. It is not materially affected by inorganic salts, excess sulfuric acid, temperature variations, or reasonable deviation from the outlined procedure.

SUMMARY

1. A method for the determination of quaternary ammonium salts and certain tertiary amines is presented.

2. The method readily lends itself to routine assays.

3. Concentrations in the vicinity of 1:4,000 of Ceepryn® Chloride, Bentyl® Hydrochloride, and similar compounds are readily assayed.

4. The method is rapid, sensitive, and, as indicated, titration volumes are reproducible to less than 0.1 ml. with standard laboratory equipment.

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A Method for Appraisal of Antacid Capacity*

By A. M. CORRENTE

A convenient method is presented for the *in vitro* evaluation of antacid preparations. This provides a measure of the antacid "capacity" of a preparation in terms of hydrochloric acid units with which the physician is familiar, and also in terms of *p*H values. By plotting the data in a standard way, the different characteristics of various antacid preparations may be demonstrated graphically. Illustrations are given of results obtained with several different types of antacids.

THE PHARMACEUTICAL CHEMIST needs a relatively simple method to demonstrate or appraise *in vitro* the characteristics of an antacid preparation, especially to show the "capacity" for neutralization of acid and the pattern of *p*H changes that can be expected after mixing an aqueous suspension with increments of acid. It is desirable that this capacity be expressed in terms of units with which the gastroenterologist is familiar. The present report describes a method which we have evolved for our own purposes and have found useful in the development of an antacid preparation. The method appears to be helpful in appraising the speed of neutralization and the pattern of *p*H fluctuations with additions of increments of acid as well as the buffering capacity. It can be applied to various individual ingredients, as well as to the finished tablets.

Various methods for the *in vitro* appraisal of antacids have been published heretofore (1–4) and, in general, these have served as a guide for our studies. As experience with our adaptation of these methods was gained, we learned to alter certain conditions so that interesting differences between various preparations could be brought to light. We omitted intentionally any efforts to reproduce *in vitro* the intermittent emptying of the stomach, because it is evident that this process is highly variable *in vivo*.

EXPERIMENTAL

Method.—Place 100 cc. of distilled water in a 400-cc. glass beaker which is in a constant temperature bath (37°). Stir the liquid continuously, adjusting the rate of movement so that particles are only gently moved about. Add the test material either as a powder or as tablets to the liquid and after ten minutes record the *p*H of the mixture.

During the next fifteen seconds add 10 cc. 0.1 *N* HCl, and after forty-five seconds of mixing, determine and record the *p*H. Allow nine minutes for further mixing and note the *p*H. Immediately add another 10-cc. quantity of 0.1 *N* HCl and after a forty-five second interval of stirring, again repeat the *p*H determination. In this manner, at what amounts to intervals of ten minutes, continue to add 10-cc. quantities of 0.1 *N* HCl until the *p*H fails to show any fall when the reading is taken after forty-five seconds of stirring following the addition of acid, or a rise after nine minutes of stirring. In other words, the end point is reached when the forty-five second-*p*H and nine minute-*p*H readings are found to be approximately the same. Usually, this is reached at *p*H 2.0 (± 0.2).

A few features of this procedure deserve special consideration. *The temperature* of the liquid is important, for a different pattern of *p*H change may be observed when the temperature is varied.

The speed of the stirring device should be adjusted so that mixing is complete, yet the action is not so violent as to disrupt mechanically tablets or the granules of the tablets, for these should be permitted to disintegrate as they will in contact with the moving liquid.

The addition of pepsin to the HCl was found to alter somewhat the pattern of response. A solution of 0.5% pepsin in 0.1 *N* HCl has been employed after initial increments of HCl alone have brought the *p*H to 6.0 or below. At this time, in order that the pepsin content be uniform, an amount of pepsin, U. S. P. equal to 0.5% of the total quantity of the test liquid in the beaker has been added. Thereafter, increments of the solution of 0.1 *N* HCl with 0.5% pepsin were used. This procedure was followed for the addition of pepsin in order to avoid destruction of pepsin which may occur in an alkaline medium, and at the same time, control the quantity of pepsin employed.

The timing of the readings of *p*H was set at intervals which portrayed the extremes of *p*H fluctuation with each increment of acid. Forty-five seconds of stirring after the addition of acid appeared to be ample to assure sufficient mixing. In the subsequent nine minutes, the buffering action of the antacid charge occurred in most instances to its fullest, since measurement of the *p*H at intervals longer than nine minutes has not shown further significant alteration of *p*H, at least with the preparations thus far tested. Conceivably, preparations slower to react would require an interval longer than nine minutes to determine the highest *p*H fluctuation to be obtained. Conversely, we have encountered rapidly reacting preparations for which an interval of five minutes has been more than sufficient to show the highest *p*H extreme.

Graphic plotting of the data. The volume of acid in cc. equals the clinical units of acid with which the gastroenterologist is familar. The points plotted for each of the forty-five-second *p*H and the nine-minute *p*H values illustrate the *p*H fluctuations that occur upon the addition of acid and thus neutralization capacity is shown.

* Received August 21, 1953, from the Laboratories of Organon, Inc., 20 Main St., Orange, N. J.
Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.

RESULTS

The foregoing method has been utilized in our development of an antacid product, in the course of which a number of standard antacid preparations were studied. Of the many graphs prepared from the data, eight, which show certain distinctive characteristics of several preparations, have been selected to demonstrate the application of this method.

The quantity of the active ingredients differed somewhat in each of these tests because we employed commercial tablets whose weights and active content varied. The Sippy powders were tested in quantities of 1 Gm. rather than the unwieldy usual clinical dose of 2.6 Gm. Commercial tablets that are intended to be swallowed were simply allowed to disintegrate as they would in the test fluid and this occurred in the first ten-minute interval. Antacid medication that is intended to be chewed (*Product B*) before swallowing was powdered in a mortar before testing. The manner and speed of disintegration of commercially available antacids affects the *p*H readings not only in the *in vitro* determinations, but also when measured by the relief of symptoms of hyperacidity and gastric distress in the ulcer patient. Unless exceptions are indicated, all of the antacid tablets studied were dropped into distilled water and permitted to disintegrate in the moving liquid controlled by the stirring apparatus. The mixture was held at a constant temperature of 37° unless changes are so indicated.

The preparations illustrated in the graphs are as follows.

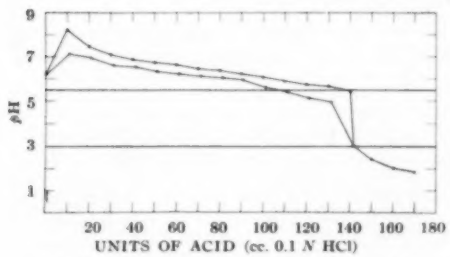

Fig. 1.—Sippy Powder No. 1 (1.0 Gm.).

sodium bicarbonate	0.77 Gm.
calcium carbonate	0.23 Gm.
	1.00 Gm.

Sippy Powders No. 1 and No. 2 (Figs. 1 and 2).—These materials show a high capacity for acid neutralization but this capacity is entirely within alkaline *p*H ranges. Although some doubt has been cast on the existence of "acid rebound secretion" after exposure of the stomach to alkaline preparations, yet the probability that it may occur has not clearly been denied especially when large amounts of alkali are used. The administration of alkali in small amounts, such as would be rapidly neutralized by the gastric pool has not been seriously questioned (5). Figures 1 and 2 illustrate the alkaline feature of the Sippy powders, although their high capacities for neutralizing acid are quite evident. They also show that the antacid charge of the Sippy powder is immediately attacked by the acid, since the *p*H recorded forty-five seconds after each addition of acid is high, indicating that a considerable quantity of the acid has already been neutralized in this short interval. In testing these powders, the nine-minute interval probably could be shortened.

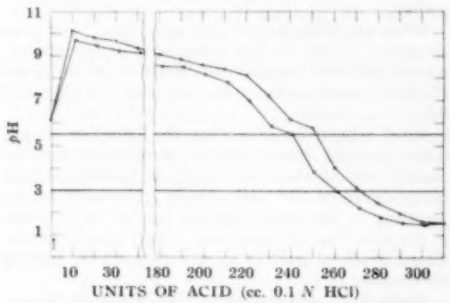

Fig. 2.—Sippy Powder No. 2 (1.0 Gm.).

sodium bicarbonate	0.5 Gm.
magnesium oxide	0.5 Gm.
	1.0 Gm.

NOTE: That portion of the graph is deleted which shows no significant *p*H change with the addition of 140 cc. of acid according to our method.

Product *A*—Dried Aluminum Hydroxide (Fig. 3).—Commercial tablets containing dried aluminum hydroxide were employed in this study. Each weighed 1.05 Gm. and contained 0.6 Gm. of active ingredient, described as $Al_2(OH)_6$. This particular ingredient showed an unexpected difference in its response when the test was carried out under different conditions of temperature or with the addition of pepsin, as is shown in Fig. 3. Early studies were made at 25° without pepsin and showed very

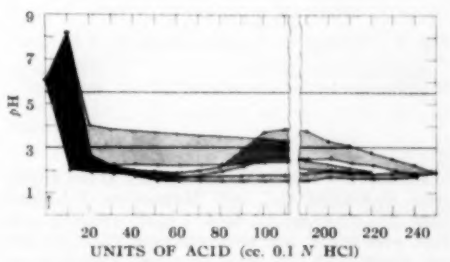

Fig. 3.—Product A, 1 tablet (1.05 Gm.).

aluminum hydroxide	0.6 Gm.

NOTE: That portion of the graph is deleted which shows no significant *p*H change with the addition of 80 cc. of acid according to our method.

X Standard method, 0.1 *N* HCl plus pepsin, 37° C.
○ Standard method, 0.1 *N* HCl plus pepsin, 25° C.
● Standard method, 0.1 *N* HCl, no pepsin, 25° C.

little acid neutralizing capacity, for the *p*H fluctuation fell below *p*H 2.0 after the addition of only 40 cc. of acid. This indicated free acid was present as shown by Töpfer's reagent, even after an additional 20 cc. In an effort to find the end point, however, further increments of acid were added, and after 90 cc. had been added, a revival of buffering capacity was revealed, as is shown in Fig. 3. When the test was repeated with pepsin added, however, we were never able to demonstrate this additional buffering action.

When the temperature was raised to 37° and pepsin added, a very definite buffering capacity was observed and this capacity was quite large, although the *p*H hovered always in the low ranges of *p*H 2.0 to 3.0.

After repeatedly observing the behavior of the substance under these different conditions, we concluded that all the tests should be performed at a temperature of 37° with pepsin. It would be interesting to determine whether further refinements in the direction of employing a more strictly reproduced artificial gastric juice would reveal further differences.

Product *B*—Magnesium Trisilicate with Aluminum Hydroxide (Fig. 4).—Commercial tablets each weighing 1.32 Gm. were employed, and the active ingredients were 0.5 Gm. of magnesium trisilicate and 0.25 Gm. of dried aluminum hydroxide gel. The tablets were powdered in a mortar, because they do not disintegrate in water and the label advises that the tablets be chewed. The results of tests as illustrated in Fig. 4 reveal that the buffering capacity of this tablet is limited, for the free acid level is reached when only 30 units of acid are added.

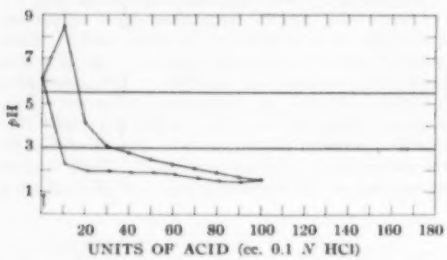

Fig. 4.—Product B, 1 tablet powdered (1.32 Gm.).

magnesium trisilicate	0.50 Gm.
aluminum hydroxide gel	0.25 Gm.
	0.75 Gm.

Product *C*—Tablets Gastric Mucin, Dried Aluminum Hydroxide Gel, and Magnesium Trisilicate (Fig. 5).—Commercial tablets each weighing 1.14 Gm. were tested, and each contained 0.16 Gm. gastric mucin, 0.25 Gm. dried aluminum hydroxide gel and 0.45 Gm. of magnesium trisilicate. This tablet shows only a moderate antacid capacity; in this instance, the free acid level is reached when slightly less than 60 units of acid are added.

Product *D*—Tablets Aluminum Hydroxide, Calcium Carbonate, and Magnesium Peroxide (Fig. 6).—Commercial tablets were employed. Each weighed 0.56 Gm. and two of these weighing 1.12 Gm. were tested. The two contained 0.288 Gm. aluminum hydroxide, 0.142 Gm. calcium carbonate, and 0.172 Gm. magnesium peroxide. This study showed that this tablet produced a rather alkaline effect, 50 units of acid or less were consumed before the *p*H was lowered to 5.5. However, the capacity was considerable, in that an additional 60 units were consumed in order to lower the *p*H to 3.0, the free acid level.

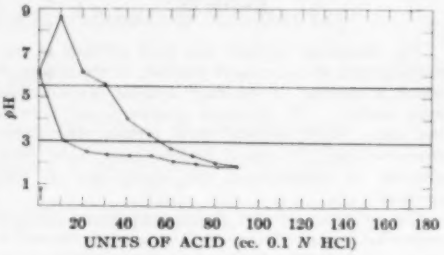

Fig. 5.—Product C, 1 tablet (1.14 Gm.).

gastric mucin	0.16 Gm.
aluminum hydroxide gel	0.25 Gm.
magnesium trisilicate	0.45 Gm.
	0.86 Gm.

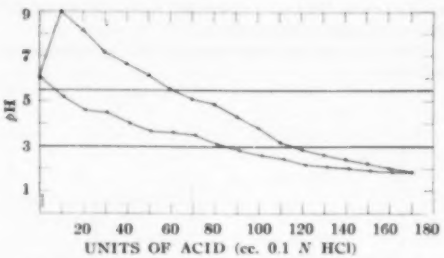

Fig. 6.—Product D, 2 tablets (1.12 Gm.).

aluminum hydroxide	0.288 Gm.
calcium carbonate	0.142 Gm.
magnesium peroxide	0.172 Gm.
	0.602 Gm.

Trevidal® Tablets—Regonol® Brand of Cyamopsis tetragonaloba Gum, Dried Aluminum Hydroxide Gel, Calcium Carbonate, Magnesium Carbonate,® and Magnesium Trisilicate (Fig. 7).—Trevidal tablets, supplied for appraisal as an unknown, were studied by this method. Each weighed 0.55 Gm. and two of these weighing 1.1 Gm. were used. The two tablets contained 0.2 Gm. Regonol®, 0.18 Gm. dried aluminum hydroxide gel, 0.21 Gm. calcium carbonate, 0.12 Gm. magnesium carbonate, and 0.3 Gm. magnesium trisilicate. The antacid ingredients are bound together in such a manner as to delay the release of a portion of the antacid charge. Therefore, it was desired to compare the capacity of finished tablets with powdered tablets and with the unprotected mixture of antacid ingredients.

Finished tablet, as illustrated in Fig. 7, showed an initial phase above *p*H 5.5, which was consumed after addition of 50 units of acid, and the total capacity

was considerable, as the free acid range is not reached until over 100 units of acid have been consumed, and remains above 2.0 until 150 units have been consumed.

Powdered tablet gave results quite comparable with those of the finished tablet, whereas the *unprotected antacid ingredients* (Fig. 7) in equivalent quantity showed a somewhat different pattern. In the latter instance, the portion of the pattern above *p*H 5.5 was more prolonged, somewhat higher, and showed less fluctuation. This difference leads us to believe that the desired binding effect was achieved. The total capacity shown in these three tests of Trevidal® is the same, however.

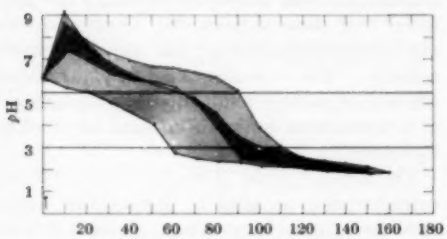

Fig. 7.—Trevidal®.

Regonol®	0.20 Gm.
calcium carbonate	0.21 Gm.
magnesium carbonate	0.12 Gm.
magnesium trisilicate	0.30 Gm.
dried aluminum hydroxide gel	0.18 Gm.
	1.01 Gm.

● Trevidal®, 2 tablets (1.1 Gm.).
X Active ingredients contained in 2 Trevidal® tablets, not granulated.

Trevidal® Tablets, Dose Repeated (1.1 Gm. + 1.1 Gm.) (Fig. 8).—After a test of this type has been completed, there may remain a quantity of mixed free and combined acid, which retains some further buffering capacity. It seemed of interest to determine the effect this would exert on repetition of the dose of Trevidal®. The test was therefore repeated, this time starting with 60 units of acid, and when the capacity of two tablets of Trevidal® was nearly consumed, two more tablets were added in a continuation of the test.

The results (Fig. 8) showed that the first 60 units of acid exhausted the antacid charge to about the same extent as was seen in Fig. 7, and the balance of the pattern was also similar. However, after the addition of two more tablets, the buffering capacity of the test liquid was such that the initial portion of this pattern is significantly altered, and the *p*H

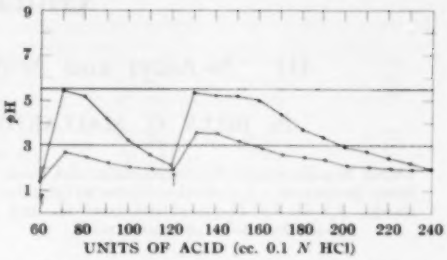

Fig. 8.—Trevidal®, 2 tablets, dose repeated.

did not rise above *p*H 5.5. The total capacity appeared to be somewhat less, indicating the presence in the test liquid of perhaps 20 units of uncombined acid.

DISCUSSION

Antacid therapy generally aims to maintain the *p*H of the stomach contents at values between *p*H 3.0 and 5.5. Below *p*H 3.0, free acid may be present (5); Hollander refers to *p*H 3.5 as the free-acidity end point (6). In the standard gastric analysis the free acid is generally taken to be the acidity observed below *p*H 3.0, in the range at which Töpfer's reagent turns red with the addition of acid, or turns yellow with the addition of base. In these graphs we have specially indicated this *p*H level as well as that of *p*H 5.5, for the pepsin is reported to be not active at *p*H above 5.0 (7).

The particular preparations which were employed to illustrate this method were chosen because they are commonly used and hence, the "capacity" as shown in terms of units familiar to the physician seems of interest. Were it not for the variable secretion of acid and the intermittent emptying of the stomach, this method could be employed as an accurate guide to estimate dosage requirements. However, hyperacidity may involve not only the secretion of an excessively acid gastric juice, but also of an exaggerated volume of this juice. Hence, the physician must adjust the dosage to each patient's requirement.

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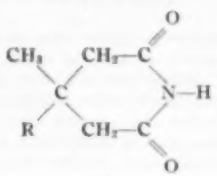
Glutarimides*

III. N-Alkyl and N-Aryl-3,3-Diethylglutarimides

By BILLY G. MALLARD† and CHARLES O. WILSON‡

Three N-substituted diethylglutarimides have been prepared: 3,3-diethyl-N-methylglutarimide, 3,3-diethyl-N-phenylglutarimide, and 3,3-diethyl-N-benzylglutarimide.

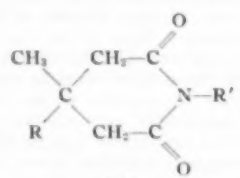
A RECENT INVESTIGATION into the synthesis and pharmacologic response of substituted glutarimides by Benica and Wilson (1, 2) revealed a single physiologically active compound among the fifteen that were synthesized. The three 3,3-dialkylglutarimides (1) I,



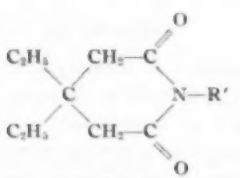
(I)

R = methyl, ethyl, or propyl

showed no pharmacologic response while all of the twelve N-alkyl-3, 3-dialkylglutarimides (2), II,



(II)



(III)

R′ = methyl, ethyl, or butyl

produced convulsions at high dosage with the exception of N-methyl-3-methyl-3-propylglutarimide which demonstrated some antimetrazol activity at 500 mg./Kg. doses.

Another series of compounds of the piperidone structure has been synthesized by Preiswerk and Schnider (3) in an attempt to produce a safe hypnotic. Of these compounds, N-methyl-2,4-dioxo-3,3-diethylpiperidione has shown hypnotic activity. The 2,4-dioxo-3,3-diethylpiperidione which does not have a substituent on the nitrogen is a nonnarcotic antitussive (Sedulon®) sold by Hoffmann-La Roche Inc.

The activity of the N-methyl-3-methyl-3-propylglutarimide prepared by Benica and Wilson and the activity of 2,4-dioxo-3,3-diethylpiperidione and N-methyl-2,4-dioxo-3,3-diethylpiperidione has prompted this investigation of a series of glutarimides which have two ethyl substituents in the 3-position, III.

Pharmacological investigations are being made on the compounds herein reported in the Department of Pharmacology, Medical School, The University of Texas at Galveston.

PROCEDURE

The formation of desired glutarimides was accomplished by initially preparing the corresponding acid, converting this to the anhydride, reacting the anhydride with ammonia or a primary amine, and then heating to split out water and any excess of ammonia or amine.

Alcoholic Ammonia.—Alcoholic ammonia was prepared in gallon lots according to Benica and Wilson (1).

2,4-Dicyano-3,3-diethylglutarimide.[1]—This product was prepared by modifying the method of Vogel (4) from diethyl ketone (100 Gm.), ethyl cyanoacetate (270 Gm.), and alcoholic ammonia (700 cc.) mixed at 0° and allowed to stand for one week. A yield of 39.8% was obtained.

3,3-Diethylglutaric Acid.—2,4-Dicyano-3,3-diethyl-glutarimide (101 Gm.) was dissolved in sulfuric acid (220 cc.) according to Vogel (4). The solution was cooled in an ice bath to prevent charring of the compound, and, after heat ceased to be evolved, was allowed to stand overnight in an open beaker. The yield was 73 Gm. (84.5%) and the crystals had a m. p. of 104–105°. Vogel (4) reports a melting point of 106°.

3,3-Diethylglutaric Anhydride.—3,3-Diethylglutaric acid (72 Gm.) was dissolved in acetic anhy-

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[1] Melting and boiling points given are uncorrected.

TABLE I.—ANALYTICAL DATA

Compound	Formula	Carbon		Hydrogen	
		Calcd.	Found	Calcd.	Found
$(C_2H_5)_2C(CH_2COOH)_2$	$C_9H_{20}O_4$	57.45	57.14	8.51	8.62
$(C_2H_5)_2C(CH_2CO)_2O$	$C_9H_{18}O_3$	63.17	63.50	8.23	8.23
$(C_2H_5)_2C(CH_2CO)_2NR$					
R = H	$C_9H_{15}O_2N$	63.92	63.82	8.87	8.75
R = CH_3	$C_{10}H_{17}O_2N$	66.08	66.12	9.28	9.10
R = C_6H_5	$C_{15}H_{19}O_2N$	73.48	73.34	7.61	7.56
R = $CH_2C_6H_5$	$C_{16}H_{21}O_2N$	74.15	74.15	8.1	8.28

dride (235 Gm.) contained in a distillation flask with a thermometer extending to the bottom of the flask. Acetic anhydride and acetic acid were distilled off until the contents of the flask reached a temperature of 175°. The flask was then cooled and the residue distilled under reduced pressure. A yield of 60 Gm. (93%) was collected at 154–158°/7 mm.

3,3-Diethylglutarimide.—3,3-Diethylglutaric anhydride (8.5 Gm.) was dissolved in dry xylene (25 cc.) in a 125-cc. flask. A stream of dry ammonia gas from a cylinder was passed through the solution for thirty minutes after which time the exothermic reaction had subsided and it was apparent that no more ammonia was reacting with the anhydride. The reaction product was then placed under a reflux condenser equipped with a Barrett moisture trap and refluxed until the theoretical amount of water (0.9 cc.) had collected. The product was recrystallized from hot 50% alcohol and only the first crop of crystals was collected. The yield was 4.6 Gm. (54.7%), the melting point 145–146°. Previous workers (5) have reported a melting point of 146–147°.

3,3-Diethyl-N-methylglutarimide.—3,3-Diethylglutaric anhydride (22.5 Gm.) was dissolved in 25 cc. of toluene contained in a 125-cc. Erlenmeyer flask. A 25% solution of methylamine (100 cc.) in water was placed in a 500-cc. flask equipped with a reflux condenser. A one-hole rubber stopper with a glass tube was inserted in the upper end of the condenser and the tube connected with a moisture trap immersed in ice. A column (30 x 2.5 cm.) was packed with sodium hydroxide pellets and connected to the moisture trap on one end to a glass delivery (3 mm. i. d. x 15 cm.) on the other. The solution of methylamine was slowly heated until the odor of methylamine could be detected coming through the caustic column. The system was allowed to purge for about one minute longer and the delivery tube was then immersed in the toluene solution of the anhydride. The rate of evolution of methylamine was controlled by the heat under the flask. A vigorous exothermic reaction occurred as the methylamine was allowed to bubble through. The bubbling was continued until it was apparent that no more methylamine was being absorbed by the anhydride. The reaction mixture was then placed under a reflux condenser with a Barrett moisture trap and refluxed until the theoretical quantity of water (2.3 cc.) had collected. The product was distilled under reduced pressure and collected at 131.8 to 132.2°/7 mm. A yield of 21.3 Gm. (89.4%) was obtained.

3,3-Diethyl-N-phenylglutarimide.—3,3-Diethylglutaric anhydride (8.5 Gm.) was dissolved in xylene (15 cc.) and added to a solution of aniline (4.6 Gm.) in xylene (15 cc.) in a 150-cc. Erlenmeyer flask. The mixture was then heated under reflux with a Barrett moisture trap interposed between the flask and the condenser. The refluxing was continued for one hour after the theoretical amount of water (0.9 cc.) had collected in the trap. The residue was diluted with toluene (50 cc.), boiled with decolorizing charcoal (2 Gm.), and filtered through a heated filter. The filtrate was allowed to cool to room temperature and then placed in an ice bath for three hours. Crystals of the product were separated by suction filtration, washed with cold benzene, and dried in a vacuum desiccator. The product had a m. p. of 124–125°. A yield of 7.3 Gm. (59.8%) was obtained.

3,3-Diethyl-N-benzylglutarimide.—3,3-Diethylglutaric anhydride (8.5 Gm.) was dissolved in xylene (15 cc.) and placed in a 150-cc. Erlenmeyer flask containing a solution of benzylamine (5.3 Gm.) in xylene (15 cc.). The mixture was then heated under reflux with a Barrett moisture trap interposed between the flask and the condenser. The refluxing was continued for one hour after the theoretical amount of water (0.9 cc.) had collected in the trap. The reaction mixture was then distilled under reduced pressure and 6.7 Gm. (51.5%) of imide was collected at 190–191°/6.5 mm.

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Some Recently Developed Chemicals as Disintegrating Agents for Compressed Tablets*

By AHMAD FIROUZABADIAN† and C. LEE HUYCK‡

Five disintegrating agents were tested for tablets, consisting of cornstarch, alginic acid, Methocel®, starch-agar mixture, and Veegum® HV. Alginic acid and cornstarch are favorable as disintegrating agents for this particular formula of sodium bicarbonate tablets. For this formula of aluminum hydroxide tablets, starch-agar and Veequm® HV were favorable disintegrators.

THE PURPOSE of this investigation is to compare some recently developed chemicals with cornstarch as disintegrating agents for compressed tablets.

The use of starch paste as a binder and dry starch as a disintegrator was found to hasten the disintegration of tablets containing an insoluble medicament (1, 2, 4, 5).

Potato starch, gelonide, and magnesium peroxide as disintegrants for tablets (3) have been compared. Gelonide was prepared by treating 10 per cent aqueous solution of gelatin with a few drops of formaldehyde solution until a glue-like consistency was attained. It was rubbed through a No. 22 screen, dried for several hours at 90°, and powdered. The disintegrants were added to an acetanilid granulation prepared with 10 per cent gelatin solution. The amount added was 1/4 Gm. per 5 Gm. granulation. One per cent talc was used as lubricant. Potato starch was found to be superior to the other two disintegrants for acetanilid tablets.

Dried bentonite was compared to cornstarch as a disintegrator for thyroid tablets (7) and it was found to be superior to cornstarch.

In a study of the variables (8) influencing the disintegration of tablets such as active constituents, binding agent, and disintegrating agent, it appeared that none of them were solely responsible for influencing the disintegration rate of the finished tablet. Disintegration was probably due to the interaction of granulating agent, disintegrating agent, and active ingredient.

In a comparison of 22 old and new tablet disintegrants (9), the new disintegrants tested were powdered sponge and citrus pulp. These substances compared favorably with cornstarch as disintegrants for tablets containing insoluble medicaments such as aspirin and aluminum hydroxide.

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† Abstracted from a thesis presented to the faculty of the St. Louis College of Pharmacy and Allied Sciences by Ahmad Firouzabadian in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
‡ Professor of Industrial Pharmacy.

EXPERIMENTAL

Four substances were compared with cornstarch as disintegrating agents for tablets of both soluble and insoluble medicaments in 10% concentrations. They are: (*a*) 5% powdered cornstarch plus 5% powdered agar, (*b*) 10% alginic acid,[1] (*c*) 10% Veegum® HV,[2] (*d*) 10% methyl cellulose 25 centipoises (Methocel®).[3] Sodium bicarbonate was selected as the soluble medicinal agent while aluminum hydroxide was selected as the insoluble medicinal agent. The formula for the five batches of 7,000 five-grain sodium bicarbonate tablets containing the various disintegrants is given in Table I.

Ten per cent acacia mucilage was used as granulating agent. The acacia was placed in a suitable container and an equal weight of warm distilled water was added to it. It was agitated for several minutes, set aside, and heated on a steam bath until dissolved. It was then strained through muslin.

A Stokes mixer was used in the preparation of the granulation. One pound of 10% acacia mucilage was found to give the mass the desirable adhering properties. To ensure exactness of procedure in the wet granulation method, the five batches of tablets were granulated at one time. Uniformity as to mixing, amount of binding agent used, size of particles, drying, screening the dry granulation, and lubrication were attained in this manner. One-tenth pound of magnesium stearate per batch was used as lubricant. A No. 10 mesh screen was used for the wet granulation on the oscillating granulator and the granules were spread on perforated trays covered with paper to dry. The granules were dried in the drying oven for forty-eight hours at 48°.

The dry granulation was forced through a No. 12 screen by means of the oscillator and the lubricant was forced through a No. 80 mesh screen before it was added to the granulation. The granulation was placed in the Stokes mixer and the lubricant was added gradually with constant agitation.

The lubricated granulation was divided into five equal parts. To each part 10% of the disintegrating agent to be tested was thoroughly mixed with the granulation in the mixer.

For compressing, the Stokes single punch E machine equipped with 13/32-inch shallow concave punches and die were used. The machine was run at a speed of 90 tablets per minute. The weight of each tablet was 5.7 grains containing 5 grains

[1] Alginic Acid was obtained from Kelco Co., 20 N. Wacker Drive, Chicago 6, Ill.
[2] Veegum® HV was obtained from the R. T. Vanderbilt Co., 230 Park Ave., New York 17, N. Y.
[3] Methocel®, brand of methylcellulose, was obtained from Dow Chemical Co., Midland, Mich.

TABLE I.—TABLE OF INGREDIENTS IN POUNDS FOR 7,000 SODIUM BICARBONATE FIVE-GRAIN TABLETS

	Starch	Starch and Agar	Alginic Acid	Veegum® HV	Methyl Cellulose
Sodium bicarbonate	5.0	5.0	5.0	5.0	5.0
Starch	0.5	0.25	...	...	...
Agar	...	0.25	...	...	...
Alginic acid	...	...	0.5	...	...
Veegum® HV	...	...	...	0.5	...
Methyl cellulose	...	...	...	...	0.5
Magnesium stearate	0.1	0.1	0.1	0.1	0.1

TABLE II.—AVERAGE DISINTEGRATION TIME[a] IN SECONDS OF THE FIRST AND SECOND BATCHES OF SODIUM BICARBONATE TABLETS CONTAINING VARIOUS DISINTEGRANTS

	First Batch	Second Batch	Mean
Starch	42.6	44	43.3
Alginic acid	11.6	12.3	11.95
Methocel® 25 c. p. s.	50	51	50.5
Starch and agar	33.3	32.3	32.8
Veegum® HV	14.3	13.6	13.9

[a] Average of three U. S. P. disintegration tests.

of sodium bicarbonate. An attempt was made to keep the hardness of the tablet constant at 5 Kg. as measured with the Monsanto Hardness Tester. Also, an attempt was made to keep the hopper filled to obtain tablets of as uniform weight as possible.

Tablets were collected at various intervals from each of the five batches for the disintegration tests. A second batch of sodium bicarbonate tablets was prepared as nearly like the first batch to obtain more disintegration data on the tablets prepared by this formula plus the various disintegrants. The disintegration tests were conducted in accordance with the U. S. P. directions (10). Three tests were made on each batch and the average time of disintegration was calculated. The average disintegration time in seconds of the first and second batches of sodium bicarbonate tablets containing the various disintegrants is given in Table II.

Aluminum hydroxide was selected as the insoluble medicament to be tested with the various disintegrants. As with sodium bicarbonate tablets, a batch containing 10% powdered cornstarch as disintegrator acted as the control. The formula for the five batches of 7,000 five-grain aluminum hydroxide tablets containing the various disintegrants is given in Table III.

Ten per cent starch paste was used as granulating agent. The cornstarch was placed in a suitable container and an equal weight of distilled water was added to it in order to make a uniform paste. The mixture was heated until a translucent mass was formed. The heat was removed and the weight of the mass was adjusted with distilled water to contain 10% starch. The processes of granulating, screening, drying, and compressing were the same as for the sodium bicarbonate tablets.

TABLE IV.—AVERAGE DISINTEGRATION TIME[a] IN SECONDS OF THE FIRST AND SECOND BATCHES OF ALUMINUM HYDROXIDE TABLETS CONTAINING VARIOUS DISINTEGRANTS

	First Batch	Second Batch	Mean
Starch	Over 1 hour	Over 1 hour	Over 1 hour
Alginic acid	1160	1087	1123.5
Methocel® 25 c. p. s.	1141	1286	1213.5
Starch and agar	720	660	690
Veegum® HV	760	730	745

[a] Average of three U. S. P. disintegration tests.

A second batch of aluminum hydroxide tablets was prepared as nearly like the first batch as possible to obtain more disintegration data on the tablets containing the various disintegrants. The tablets were tested for disintegration by the U. S. P. test (10). Three tests were made on each batch and the average time of disintegration was calculated. The average disintegration time in seconds of the first and second batches of aluminum hydroxide tablets containing the various disintegrants is given in Table IV.

DISCUSSION

The fact that the time of disintegration of sodium bicarbonate tablets prepared by this formula was below 50 seconds while the time of disintegration of aluminum hydroxide tablets prepared by this formula was over 500 seconds is noteworthy. The results would indicate that the statement "disintegration is often influenced by the nature of the medicament present" (6, 12) is true.

TABLE III.—FORMULA IN POUNDS FOR 7,000 FIVE-GRAIN ALUMINUM HYDROXIDE TABLETS CONTAINING VARIOUS DISINTEGRANTS

	Control	Starch Agar	Alginic Acid	Veegum® HV	Methocel®
Aluminum hydroxide	5.0	5.0	5.0	5.0	5.0
Powdered sucrose	0.1	0.1	0.1	0.1	0.1
Starch	0.5	0.25	...	...	...
Agar	...	0.25	...	...	...
Alginic acid	...	...	0.5	...	...
Veegum® HV	...	...	...	0.5	...
Methocel® 25 c. p. s.	...	...	...	...	0.5
Magnesium stearate	0.1	0.1	0.1	0.1	0.1

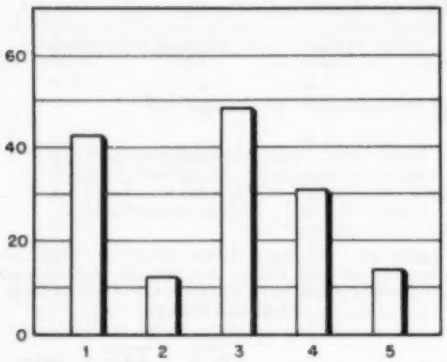

Fig. 1.—Comparison of the average disintegration time in seconds of the sodium bicarbonate tablets containing various disintegrants.
1—Corn starch. *2*—Alginic acid. *3*—Methocel®. *4*—Starch and agar. *5*—Veegum® HV.

Aluminum hydroxide tablets made by this formula containing 10% cornstarch as disintegrator did not pass the U. S. P. standards for disintegration. These tablets are therefore unsatisfactory. It is possible that the disintegration time of these tablets could be increased by reducing the size of the granules before compression. This could be accomplished by using a screen finer than a No. 12 for reducing the size of the granules before compression. Generally, for a 5-grain tablet a No. 14 screen (5) is recommended, but a No. 12 screen was used in this case to keep the fine particles at a minimum. An excess of fine particles causes tablets to cap, laminate, chip, and vary in weight or thickness (13).

For sodium bicarbonate tablets prepared by this formula, alginic acid and Veegum® HV proved to be the best disintegrators. The results with alginic acid are in agreement with previous work (11). The fact that Veegum® HV produced a slightly discolored tablet limits its usefulness, but it can be used in smaller amounts than that used and in colored or coated tablets.

For aluminum hydroxide tablets prepared by this formula, starch agar mixture and Veegum® HV gave the shortest disintegration time. Here again both disintegrators slightly discolored the tablets. Methocel® 25 c. p. s. and alginic acid are satisfactory disintegrators for aluminum hydroxide tablets with Methocel® favored because it costs less than alginic acid.

SUMMARY AND CONCLUSIONS

Of the five disintegrating agents tested for tablets, namely cornstarch, alginic acid, Methocel®, starch-agar mixture and Veegum® HV—alginic acid and Veegum® HV compare favorably as disintegrating agents for this particular formula of sodium bicarbonate tablets. Due to the slight discoloration of tablets containing 10 per cent Veegum® HV, alginic acid and cornstarch are recommended as disintegrants.

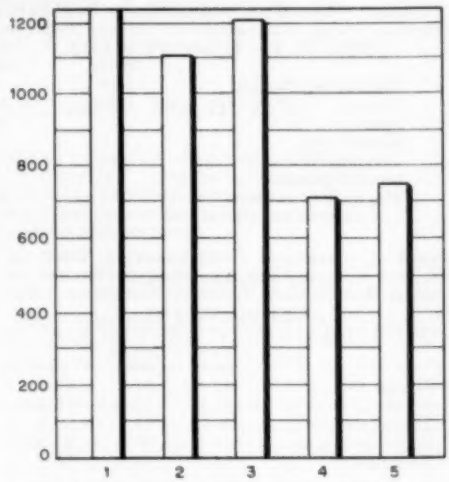

Fig. 2.—Comparison of the average disintegration time in seconds of the aluminum hydroxide tablets containing various disintegrants.
1—Cornstarch. *2*—Alginic acid. *3*—Methocel®. *4*—Starch and agar. *5*—Veegum® HV.

With aluminum hydroxide tablets prepared by this particular formula, cornstarch is worthless as a disintegrating agent. The tablets containing the starch agar mixture and Veegum® HV disintegrated in the shortest time of the five disintegrators tested. These disintegrators cannot however be recommended in 10 per cent quantities for aluminum hydroxide tablets because they discolor the tablets slightly. It is possible that an amount less than 10 per cent would be satisfactory. The starch agar mixture and Veegum® HV may be used as disintegrating agents for colored or coated tablets. Alginic acid and Methocel® 25 c. p. s. are preferred as disintegrants for this particular formula of aluminum hydroxide tablets.

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The Effect of the Monohydroxy Benzoic Acids on the Respiration of Rat Brain Homogenates*

By CHANDULAL A. PATEL and HAROLD C. HEIM

Of the isomeric hydroxy benzoates only the ortho-isomer (salicylic acid) shows analgesic antipyretic activity. A study was made of the effects of these acids on the respiration of rat brain homogenates. Respiration in the presence of glucose or pyruvate was markedly stimulated by 0.015 *M* salicylate, but not by the *meta*- nor the *para*-hydroxy benzoates in the same concentration. Respiration in the presence of glutamate was stimulated somewhat by all three hydroxy benzoates. When succinate was used as the substrate the respiration was inhibited by salicylate in concentrations of 0.015 *M* and 0.001 *M*. Neither the *meta*- nor the *para*-hydroxy benzoates produced significant effect. The respiration in the presence of lactate was slightly inhibited by all three isomeric hydroxy benzoates in 0.015 *M* concentration. Endogenous respiration was inhibited by salicylate to a greater extent than by the *meta*- or *para*-hydroxy benzoates.

IT HAS BEEN KNOWN for many years that certain derivatives of salicylic acid exert an antipyretic analgesic effect and it is now quite commonly felt that this effect is, at least in part, because of an action exerted by these compounds on the thalamus and the hypothalamus. It has also been established that derivatives of *meta*- and *para*-hydroxy benzoic acids are devoid of antipyretic analgesic effects.

In recent years a great deal of work has been reported showing that many drugs interfere with cellular respiratory processes, *in vitro* (1–3). Numerous attempts have been made to explain the mechanism of action of certain drugs on the basis of the effects of the drugs on cellular respiratory enzyme systems.

Ozu (4) in 1940 reported that large amounts of sodium salicylate promote glycogenolysis and hyperglycemia in rabbits. Lutwak-Mann (5) injected large doses of sodium salicylate into rats and observed no immediate, significant changes in blood sugar levels. From four to seven hours after administration of the salicylate, however, she observed that the liver glycogen value dropped from 28.6 per cent to about 0.2 per cent. Lutwak-Mann (6) also studied the effect of salicylates on certain enzyme systems and reported that the activity of xanthine oxidase, carboxylase, and indophenol oxidase was inhibited by salicylate. Glucose fermentation by yeast and the dismutation between hexose diphosphate and pyruvate also were inhibited. Bernheim (7) found that the addition of 1 milligram of sodium salicylate to a culture of bovine tubercle bacilli more than doubled the oxygen uptake whereas the *meta*- and *para*-hydroxy benzoic acids elicited no such effect. Ivanovics (8) reported that the inhibitory effect of salicylate on bacterial growth is antagonized by pantothenate, and Martin (9) has suggested that the analgesic effects of the salicylates might possibly be due to an interference with the utilization or synthesis of pantothenate.

Because of the relative pharmacologic inertness of the *meta*- and *para*-hydroxy benzoic acids, it was decided to compare the effects of these acids with those of salicylic acid on certain respiratory processes of brain tissue.

EXPERIMENTAL

Adult Sprague-Dawley rats of both sexes were killed by decapitation after which the brains were immediately removed and homogenized in cold 0.067 *M* phosphate buffer, *p*H 7.4, in a glass homogenizer. The hydroxy benzoic acids and the substrates were dissolved in 0.067 *M* phosphate buffer and the *p*H was adjusted to 7.4 before use.

Conventional manometric methods were used throughout this study. Conical vessels of approximately 15-cc. capacity were used and the oxygen uptake was determined according to the direct method of Warburg. The main compartments of each flask contained 0.3 cc. of 0.14 *M* substrate, 0.3 cc. of 10^{-4} *M* cytochrome C, homogenate equivalent to 0.100 Gm. fresh brain tissue, and 0.067 *M* phosphate buffer, *p*H 7.4. The side arm contained 0.3 cc. of 0.14 *M* hydroxy benzoic acid, and the center well contained 0.2 cc. of 10% KOH together with a small piece of Whatman No. 41 filter paper. The final volume of each flask was 3.0 cc., the gas phase was air, and the temperature of the bath was 37°. After a fifteen-minute equilibration period the manometers were adjusted to the reference point, the stopcocks were closed, and the contents of the side arm were tipped into the main vessel. The duration of each experiment was seventy-five minutes.

At the end of each experiment the contents of the main compartment of each flask were carefully withdrawn and centrifuged after which the *p*H was determined with a glass electrode instrument. This procedure was followed in order to certify that

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Presented to the Scientific Section, A. Ph. A., Salt Lake City meeting, August, 1953.

the *p*H had not changed during the course of the experiments.

With each experiment controls were run in which the effect of the hydroxybenzoates on the endogenous respiration was determined. In the controls the substrate was replaced by 0.067 *M* phosphate buffer. The endogenous respiration in the absence of the hydroxybenzoates also was determined during the course of each experiment.

RESULTS AND DISCUSSION

The effects of the hydroxybenzoates on endogenous respiration and on respiration in the presence of the various substrates are shown in Table I. The oxygen uptake due to added substrate was determined by subtracting the endogenous oxygen uptake from the total oxygen uptake. The stimulation (+) or inhibition (−) of this corrected oxygen uptake by the hydroxybenzoates is reported in Table I.

Respiration in the presence of succinate was inhibited about 50% by salicylate but was not significantly affected by the *meta*- or the *para*-isomer. Because succinate is the substrate most rapidly utilized by brain it was decided to perform experiments in which the effect of 0.001 *M* hydroxybenzoate was determined upon respiration in the presence of succinate. The results are shown in Table I and indicate that 0.001 *M* salicylate produced an inhibition of respiration of about 20%. Neither the *meta*- nor the *para*-isomer produced any appreciable effect. A slight inhibition of respiration was observed with all three hydroxybenzoates when lactate was used as the substrate.

Some inhibition of endogenous respiration was observed with all three of the hydroxybenzoates but in every instance the inhibition produced by salicylate was found to be slightly greater than that produced by either the *meta*- or *para*-isomer. Levy (10) reported that physostigmine salicylate stimulated the respiration of rat brain, and Ellis (11)

TABLE I.—THE EFFECT OF THE HYDROXYBENZOATES ON RESPIRATION OF RAT BRAIN HOMOGENATES

Substrate	Benzoate	Concentration of Benzoate, *M*	Total O_2 Uptake, cu. mm.	Endogenous O_2 Uptake, cu. mm.	O_2 Uptake Due to Added Substrate, cu. mm.	Stimulation or Inhibition, %
None	None	...	...	49.6	...	...
Pyruvate	None	...	106.1	49.6	56.5	...
Pyruvate	*m*-Hydroxy	0.015	98.4	40.4	58.0	+3
Pyruvate	*p*-Hydroxy	0.015	100.6	39.8	60.8	+7
Pyruvate	Salicylate	0.015	128.1	35.0	93.1	+64
None	None	...	...	44.4	...	...
Glucose	None	...	62.1	44.4	17.7	...
Glucose	*m*-Hydroxy	0.015	57.2	38.7	18.5	+4
Glucose	*p*-Hydroxy	0.015	57.3	40.0	17.3	−2
Glucose	Salicylate	0.015	78.8	36.2	42.6	+140
None	None	...	...	46.1	...	...
Glutamate	None	...	73.1	46.1	27.0	...
Glutamate	*m*-Hydroxy	0.015	61.1	38.7	22.4	+17
Glutamate	*p*-Hydroxy	0.015	61.5	41.4	20.1	+26
Glutamate	Salicylate	0.015	54.3	35.0	19.3	+29
None	None	...	...	40.9	...	...
Lactate	None	...	78.0	40.9	37.1	...
Lactate	*m*-Hydroxy	0.015	72.7	37.8	34.9	−6
Lactate	*p*-Hydroxy	0.015	70.7	37.7	33.0	−11
Lactate	Salicylate	0.015	62.3	29.0	33.3	−11
None	None	...	...	49.3	...	...
Succinate	None	...	514.4	49.3	465.1	...
Succinate	*m*-Hydroxy	0.015	458.8	37.6	421.2	−9
Succinate	*p*-Hydroxy	0.015	502.2	39.6	462.6	0
Succinate	Salicylate	0.015	272.0	34.4	237.6	−49
None	None	...	...	41.7	...	...
Succinate	None	...	600.0	41.7	558.3	...
Succinate	*m*-Hydroxy	0.001	588.0	39.5	548.5	−2
Succinate	*p*-Hydroxy	0.001	580.7	40.0	540.7	−3
Succinate	Salicylate	0.001	491.0	38.1	452.9	−20

Table I shows that the respiration in the presence of glucose was more than doubled by salicylate, while neither the *meta*- nor the *para*-isomer produced any significant effect. Salicylate produced a 64% stimulation of respiration when pyruvate was used as the substrate but neither the *meta*- nor the *para*-isomer significantly effected respiration. With glutamate as the substrate all three of the hydroxybenzoates produced some stimulation of respiration. The stimulation produced by salicylate, however, was not found to be markedly greater than that produced by the *meta*- or the *para*-isomer.

suggested that the stimulation observed by Levy might be due to the metabolism of salicylate by brain. In view of the stimulation of respiration observed when salicylate is added to homogenates respiring in the presence of glucose or pyruvate, it appears possible that salicylate is oxidized in the presence of these two substrates.

In order to determine whether pantothenate would reverse the effects produced by salicylate, experiments were performed in which pantothenate was added to homogenates respiring in the presence of some of the substrates listed in Table I. Pre-

liminary results indicate that not all the effects produced by salicylate are reversed by added pantothenate. Further work on this phase of the problem is now in progress.

SUMMARY

1. The respiration of rat brain homogenate, with succinate as the substrate, is inhibited by salicylate but is not appreciably inhibited by either *meta*- or *para*-hydroxybenzoate. All three isomeric hydroxybenzoates produce a very slight inhibition of respiration when lactate is used as the substrate.

2. The respiration of rat brain homogenate, with pyruvate or glucose as the substrate, is stimulated by salicylate but not by the *meta*- nor the *para*-hydroxybenzoates. Some stimulation was produced by all three hydroxybenzoates when glutamate was used as the substrate.

3. The endogenous respiration of rat brain homogenate was inhibited by the hydroxybenzoates but the greatest inhibition was produced by salicylate.

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Note

A Note on a Simple Method of Observing Uterine Activity *in Situ**

By H. C. FERGUSON,† T. S. MIYA,‡ and L. D. EDWARDS§

A NEW METHOD of recording uterine activity was devised and employed while evaluating the oxytocic activity of a principle isolated from *Caulophyllum thalictroides* (1). It was felt that it might be helpful to present the method in greater detail.

Albino rats, 175–250 Gm., in late estrus were found to be most suitable. The animal is anesthetized by a subcutaneous injection of 1 cc./Kg. of a 25% urethan solution containing 30 mg./cc. of pentobarbital sodium. The rat is fastened dorsally to an operating board and the vagina is filled with warm Locke-Ringer's solution by means of a medicine dropper. The large flanged end of a medicine dropper is used as a cannula and is then inserted into the vagina and tied in place with thread. The cannula is filled with warm Locke-Ringer's solution and connected by a short rubber tubing to a glass T-tube. The opposite end of the T-tube is clamped off and the upright stem is connected to a sensitive tambour for recording uterine

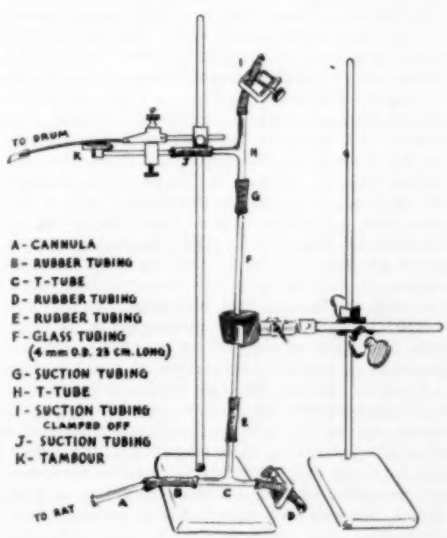

Fig. 1.—Apparatus employed.

* Received November 5, 1953, from the College of Pharmacy, University of New Mexico, Albuquerque, N. M.
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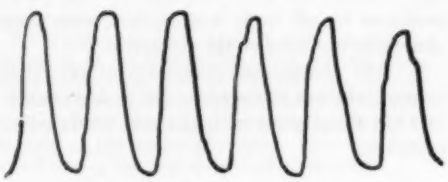


Fig. 2.—Normal contractions.

movement (Fig. 1). Pressure is applied to the system with a syringe. The needle is inserted into the short rubber tube *B* and the pressure is measured by observing the height of solution in the upright stem. An optimal pressure for each rat was necessary to set off the peristaltic type movements which in turn are detected by the meniscus changes in the upright stem. When this movement is observed, the system is connected to the tambour. The iliac vein is isolated for injections.

This preparation does not show dramatic tonus changes, but shows the movements of the uterine horns as they lie in their normal position. A typical recording is shown in Fig. 2.

SUMMARY

A simple procedure to record uterine activity *in situ* has been described.

REFERENCE

(1) Ferguson, H. C., and Edwards, L. D., unpublished report, 1952.

Book Notices

Maladies médicamenteuses d'ordre thérapeutique et accidentel. (Diseases due to the Side Effects of Drugs of Therapeutic or Accidental Origin.) By CLAUDE ALBAHARY. Masson & Cie, Paris, 1953. 752 pp. Illustrated. 17.5 x 25 cm. Price: paper bound, 4,400 *fr.*, hard cover, linen, 5,000 *fr.*

This book, following so shortly after L. Meyler's *Side Effects of Drugs* (Elsevier, 1950), cannot escape comparison with the earlier work. Meyler is a most useful, practical compendium, affording a quick survey and thorough bibliographies serving as guides to original sources. Albahary is more ambitious, and his book approaches "Handbook" volume. Its perusers will generally find it unnecessary to do any further reading on the pathologic conditions described therein. As he—like Meyler—has arranged his material according to therapeutic and at the same time, pathological agents, repetition in the description of pathological states has not been altogether avoided; but, while this increases the book's volume, it facilitates its use as a reference work. For each product, a short historical sketch is presented; this is followed by description of its metabolic properties *in vivo* and its essential pharmacodynamics as *a priori* information on possible risks.

Albahary stresses the legal implications inherent in the development of medicine from art to science during the last 60 years; a French court decision is cited which imposes upon physicians "the obligation to study the temperament, general condition, and resistance of their patients, including all individual characteristics shown, and to reassure themselves that they can tolerate any toxic substance which they administer to them even though they may not prescribe more than the usual dosage." On the basis of that legal opinion, it is, of course, indispensable to the physician to be aware of such harbingers of danger as the precursory symptoms of erythrodermia or the first indications of agranulocytosis; careful descriptions of these are included in the numerous clinical reports. The work is carefully documented and well illustrated. It is important as the first large-scale treatment of its subject.

Methoden der Organischen Chemie. (*Houben-Weyl*), 4th ed., Vol. II (Analytische Methoden). EUGEN MÜLLER, Editor. Georg Thieme Verlag, Stuttgart, Germany, 1953. (Agents for the U. S.: Grune and Stratton, 381 Fourth Avenue, New York, N. Y.) xxiv + 1070 pp. 18 x 26.5 cm. Price *DM* 139.

The eighth volume, Sauerstoffverbindungen III, was described in the Book Notices Section of THIS JOURNAL, **42,** 643(1953). In this review the plan for issuing about 13 additional volumes was described. The second volume is now available either from the publisher in Germany or from his agent in the United States. This volume of the highly esteemed project started by Weyl more than 50 years ago, covers analytical methods in organic chemistry. In the earlier editions of *Houben-Weyl* this material was widely dispersed in the various chapters. Its compilation in a single volume increases greatly the usefulness of the information presented. The book, however, is not limited to a description of analytical methods supplementing the special methods, but constitutes by itself an independent treatise on the most important analytical methods of organic chemistry.

The book is divided into two main parts. In the first part, elementary analysis is described in minute detail and in a manner designed to enable the chemist to work directly from the instructions given and without the necessity of referring to the original literature or to supplementary books on the subject. The first chapter in Part I is followed by one on the analytical determination of the most important functional atomic groups and classes of compounds.

In Part II, which includes five chapters, the methods applicable to the determination of gases are covered in great detail in the first chapter. The next chapter is devoted to the determination of melting points and boiling points, and in the third chapter the thermo-analysis and determination of molecular compounds are competently presented. A chapter on chromatographic methods of analysis is especially noteworthy and of considerable interest to the pharmaceutical chemist. Nearly all methods and applications of chromatography are discussed in sufficient detail to serve as a guide either to one experienced in these methods or to the beginner. The final chapter is devoted to methods of analysis of mixtures of solvents.

Volume II of *Houben-Weyl* is documented throughout with the literature references appearing as footnotes on each page where cited. The index is quite complete and adds greatly to the usefulness of the book. The printing is excellent, the binding is of a durable quality, and the format is unusually well-planned and designed.

A Policy for Scientific and Professional Manpower. By the National Manpower Council. Columbia University Press, New York, 1953. xvi + 263 pp. 16 x 24 cm. Price $4.50.

This book is divided into two parts, the first of which is a statement by the National Manpower Council, presenting recommendations to the nation for developing more reliable knowledge about our human resources; for strengthening institutions that educate and train scientists and professionals; for maintaining a continuous flow of students into institutions of higher education; for expanding the opportunities for capable young people to secure advanced training; and for utilizing more effectively the available supply of scientific and professional manpower. The recommendations include suggestions to the President, the Secretary of Defense, the universities, business, the professional societies, and the public.

The second part of the book was prepared by the Research Staff under the direction of Dr. Eli Ginzberg, Director of Research, and Dr. Henry David, Executive Secretary of the Council. It is a unique and comprehensive review of the facts and issues upon which the recommendations are based. These facts were compiled and evaluated with the help of top experts—the staffs of professional societies and other noted specialists in engineering, teaching, medicine, and physics. Part II begins with a history of the personnel in the sciences and the professions, depicting different rates of increase resulting from changing economic and social conditions. It explains and evaluates, both from the long-run and short-run points of view, the shortages that now exist. There is a chapter on how the Armed Forces have used and misused this highly trained group of individuals; a discussion of the deflecting of scientists into applied research work; a critical evaluation of the fact that the engineering schools are turning out the sought-after business managers for our complex economy; and an analysis of the country's reserves for scientific and professional training.

For every citizen, here is an over-all picture of what is happening to scientific and professional personnel. For everyone concerned with the future security of the United States, this book supplies facts and policies—the indispensable tools for straight thinking and future action.

Pharmacology and Therapeutics—A Textbook for Students and Practitioners of Medicine. By ARTHUR GROLLMAN. 2nd ed., revised and enlarged. Lea & Febiger, Philadelphia, 1954. 866 pp. 127 illustrations. 16 x 24 cm. Price $10.

The purpose of this book is to furnish practitioners and students a means of acquiring a sound foundation for the rational and scientific use of drugs. Dr. Grollman's experience as a teacher, writer, and investigator in pharmacology as well as in clinical medicine, should qualify him to present a practical as well as theoretical approach to the subject.

The actions, indications, toxic effects, dosages and methods of administration of all of the drugs official in the U. S. P. and the *British Pharmacopoeia* are described in detail. Many drugs official in the *National Formulary* are also similarly discussed, but their status as N. F. drugs is ignored. It is difficult to rationalize this peculiar treatment of N. F. drugs which may result in creating the false impression that U. S. P. and B. P. drugs are sacrosanct, whereas drugs listed in the N. F. are inferior. It is interesting to note the large number of B. P. drugs listed in Grollman's book which are also official in the N. F. If it is desirable to indicate the official status of some drugs, the author should follow the practice for all drugs mentioned. Emphasis is on the pharmacology of therapeutically important drugs but those of purely pharmacologic interest have not been neglected. In the index, all are listed by their common as well as U. S. P. and B. P. names. They are also included under the effects and symptoms which they induce, and the disorders in which they are used.

The enlarged *second edition* has been edited to include many of the newly introduced drugs which have become available since the appearance of the earlier edition. There is a new chapter on the pharmacology of the kidney, with an evaluation of diuretic drugs used in medicine. Advances in treatment are presented in the chapters on antimalarials and amebacides; and new discoveries in the medical treatment of malignancy are contained in the chapter on drugs acting on the blood. Also rewritten is the chapter on water and inorganic salts, with a clear exposition of the role of salt and water metabolism in health and disease. The useful epitome of prescription writing is one of the several helpful features. There are 23 new and original illustrations. Because most of the modern drugs have been introduced only recently, Dr. Grollman's work constitutes a useful addition to libraries including pharmacology within their framework. It reflects the many therapeutic developments which have taken place in the past few years.

Handbook of Probability and Statistics With Tables. By RICHARD STEVENS BURINGTON and DONALD CURTIS MAY. Handbook Publishers, Inc., Sandusky, Ohio, 1953. 332 pp. 14.5 x 20 cm. Price $4.50.

The authors of this handbook have succeeded in their effort to present in a small and compact volume the essentials of the theories of probability and statistics as well as working rules for practical application. This volume is a companion book to Burington's *Handbook of Mathematical Tables and Formulas.* Both together are intended to serve the needs of students and research workers who use statistics and mathematics in a wide variety of fields.

The first part of the book contains chapters on elementary probability theory, probability distribution in one, two, or more dimensions including binomial, Poisson, and normal distributions. It treats, among others, analysis of pairs of measurements, regression theory and time series, statistical inference, analysis of variance, finite differences, and sequential analysis.

The second part contains tables of distributions, of mathematical functions and other quantities frequently used, and tables of logarithms. There is also a brief selection of references and, besides the usual index of author and subject, an index of statistical and mathematical symbols and an index of Greek symbols as used in statistics.

Chemical Methods in Industrial Hygiene. By FREDERICK H. GOLDMAN and MORRIS B. JACOBS. (Vol. 3 of *Interscience Manuals.*) Interscience Publishers, Inc., New York, 1953. 274 pp. 13 x 18.5 cm. Price $3.75.

This small book represents the third in a series of manuals published for the purpose of providing a straightforward description of laboratory procedures and methods for the evaluation and recording of experimental results. In this particular manual an attempt has been made to describe in detail many of the analytical methods available for the examination of materials, substances, and samples encountered in the field of industrial hygiene. Emphasis has been placed on methods which are usually performed in the chemical laboratory. Thus procedures involving the use of field instruments or special laboratory apparatus such as X-ray diffraction, polarography, spectroscopy, and petrology have been omitted. The book includes nine chapters, a bibliography, an appendix, and an adequate index. The first chapter is devoted to an introduction and the important subject of sampling. This is followed by chapters on reagents and standard solutions, determination of size distribution of dusts and powders, free silica, inorganic compounds and gases, metals, general methods for volatile solvents, organic solvents, and miscellaneous gases, vapors, and mists.

BOOKS RECEIVED

Diabetic Care in Pictures—Simplified Statements with Illustrations Prepared for the Use of the Patient. By HELEN ROSENTHAL and JOSEPH ROSENTHAL. J. B. Lippincott Co., Philadelphia, 1953. 2nd ed. 164 pp. Illustrated. 16 x 23 cm. Price $3.00.

Pharmacy and Medicine in Old Edinburgh. By C. G. DRUMMOND. (A paper read at a meeting of the Scottish Society in Edinburgh on January 21, 1953.) London, The Pharmaceutical Press, 17 Bloomsbury Sq., W. C. 1, 1953. 35 pp. Paper bound. Illustrated. 14 x 22 cm. Price 2*s.*6*d.*

British Veterinary Codex, 1953. Published by Direction of the Council of the Pharmaceutical Society of Great Britain. The Pharmaceutical Press, London, 1953. xxiii and 737 pp. 15.5 x 23.5 cm. Price 45*s* (plus 1*s.* postage).

Detergency Evaluation and Testing. By JAY C. HARRIS. Interscience Publishers, Inc., New York, 1954. 210 pp. Illustrated. 13 x 18.5 cm. Price $3.75.

Aportacion de la Escuela Espanola a la Tecnica Endobronquial. (Contribution of the Spanish School to Endobronchial Techniques.) By S. GARCIA-VICENTE. Royal National Academy of Medicine, Madrid, 1953. 41 pp. Illustrated. 17 x 24 cm. Paper-bound.

The Indian Pharmaceutical Codex. Vol. I. (Indigenous Drugs.) By R. MUKERJI. Council of Scientific and Industrial Research, New Delhi, 1953. x and 431 pp. 17 x 25.5 cm. Price *Rs.* 12/–.

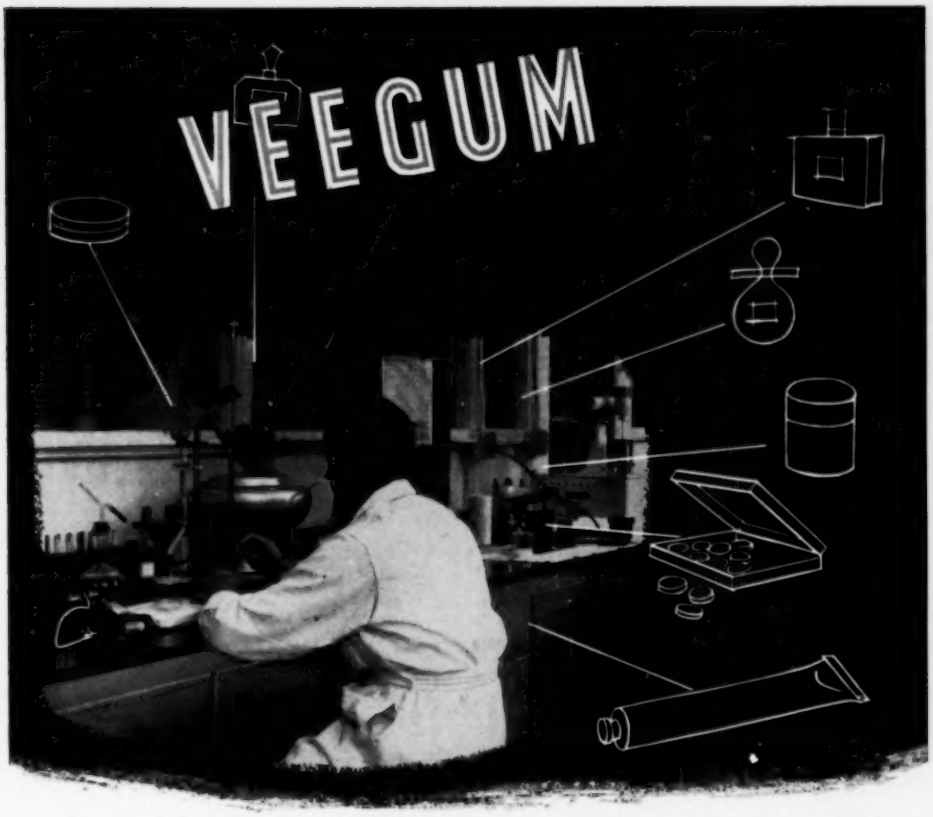
Elementary Introduction to Molecular Spectra. By BØRGE BAK. North-Holland Publishing Co., Amsterdam (Interscience Publishers, New York), 1954. 125 pp. 14.5 x 21.5 cm. Price $2.90.

Figures Pharmaceutiques Françaises—Notes Historiques et Portraits, 1803–1953. (Famous French Pharmacists.) Published by Masson & Cie, Paris, 1953, under the auspices of the Friends of the Faculty of Pharmacy, Paris, with introduction by René Fabre, Dean of the Faculty of Pharmacy of Paris. 268 pp. 44 woodblock prints. 19.5 x 28.5 cm. Luxury edition, numbered copy.

Dictionary of Organic Compounds—The Constitution and Physical and Chemical Properties of the Principal Carbon Compounds and Their Derivatives, together with the relevant Literature References. In four volumes. Second edition. SIR IAN HEILBRON and H. M. BUNBURY, Editors-in-Chief. Oxford University Press, New York, 1953. Vol. I: Ababole—Cytosine, 654 pp. Vol. II: D. A.—Hystazarin, 845 pp. Vol. III: Ibogaine—NW-Acid, 838 pp. Vol. IV: Obaculactone—Zymosterol, 694 pp. 21.5 x 27 cm. Price $78.00 the set.

The Pharmaceutical Pocket Book. 16th ed. Published by direction of the Council of the Pharmaceutical Society of Great Britain. The Pharmaceutical Press, London, 1953. 422 pp. 13 x 19.5 cm. Price 18*s.*6*d.*

Quantitative Analysis via Functional Groups. 2nd ed. By SIDNEY SIGGIA. John Wiley & Sons, New York (Chapman & Hall, Ltd., London), 1954. 227 pp. 14.5 x 22 cm. Price $5.00.



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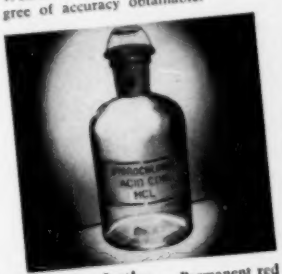

Reagent Bottles — Permanent red glass labels. Interchangeable stoppers. High chemical stability protects standard solution. Special labels available.

Pipettes — Maximum utility, readability, accuracy for long service. Never affected by repeated wet or dry sterilization. Feature smooth delivery flow.

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Corning means research in Glass